PATENT
Application No.: 10/652,745
Attorney Docket No.: 048968-117961
Via EFS-Web

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of:	
	Group Art Unit: 1617
SCHASTEEN *et al.*	
	Examiner: S. Kantamneni
Application No.: 10/652,745	
Filed: August 29,2003	
	Confirmation No. 1765
For: ANTIMICROBIAL COMPOSITIONS	

Attention: Mail Stop Appeal Brief-Patents
Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Sir:

APPEAL BRIEF UNDER BOARD RULE §41.37

In support of the Notice of Appeal filed May 23, 2008, and 37 C.F.R. § 41.37, Appellants present this brief and hereby authorize the Commissioner to charge any and all extensions or fees that may be required to Deposit Account No. 50-1662. Submitted herewith is the brief fee of $510.00 as required under 37 C.F.R. § 41.20(b)(2). Also submitted herewith is a Petition for a one-month extension of time and the $120.00 fee, which extends the period of response from July 23, 2008 to August 23, 2008.

This Appeal responds to the Final Action mailed December 11, 2007, which resulted in final rejection of claims 75, 77-104, and 113-133.

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I. REAL PARTY IN INTEREST

Novus International, Inc., is the real party in interest, as indicated by the assignments in its name, recorded at Reel 015330, Frame 0531, and Reel 016014, Frame 0275.

II. RELATED APPEALS AND INTERFERENCES

Appellants are unaware of any pending appeals or interferences that may directly affect or be directly affected by, or have a bearing on, the Board's decision in the pending appeal.

III. STATUS OF CLAIMS

Claims 75, 77-104, and 113-133 are pending in this application.

Claims 75, 77-104, and 113-133 are have been finally rejected by the Examiner, and Appellants appeal the rejection of those claims. In accordance with 37 C.F.R. 41.37 (c)(1)(viii), a clean copy of the claims on appeal are set forth in full in the Claims Appendix to this brief.

IV. STATUS OF AMENDMENTS

An Amendment after final rejection was filed on April 11, 2008, amending claims 75, 98, 99, and 104. The amendments were made to correct claim informalities and place the rejected claims in better form for appeal under 37 C.F.R. §1.116 (b)(2). In the amendments, the language "and an acceptable diluent, adjuvant or excipient" was removed from claims 75, 98, and 99. The language had been previously rejected by the Examiner as being vague, and was deleted to better define the metes and bounds of the claimed invention. In addition, the language of claim 104 was amended to clarify antecedent basis by referring to "the food" instead of "said animal food." The amendments were not entered by the Examiner, as indicated by the Advisory Action mailed on May 14, 2008. According to the Advisory Action, the proposed amendment(s) filed after

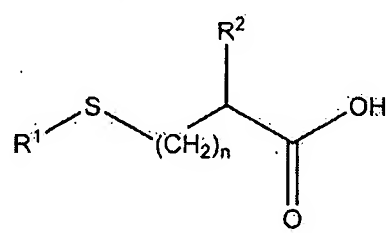
the final rejection allegedly raised new issues that would require further consideration and/or search.

No amendments to claims 77-97, 100-103, and 113-133 have been filed after the final rejection.

V. SUMMARY OF CLAIMED SUBJECT MATTER

The present invention relates to methods for inhibiting or killing microbes in water and food, including human food, livestock food, pet food, or other animal food.[1] The present invention also relates to treating food or water with a composition comprising a compound of formula (I) and one or more organic acids.[2] Independent claims 75, 98, and 99 are pending and are supported by the specification as follows.

The claimed methods include at least two organic acids selected from the group consisting of formic acid, butyric acid, fumaric acid, lactic acid, benzoic acid, and propionic acid.[3] The claimed methods also include a compound of formula (I),[4] the structure of which is indicated below.[5]



I

wherein R^1 is an alkyl group having from one to four carbon atoms;[6]
n is an integer from 0 to 2;[7]
R^2 is selected from the group consisting of hydroxy, amino, $-OCOR^3$, or $-NHCOR^3$;[8]

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[1] See, *e.g.*, specification at page 1, lines 10-26.
[2] See, *e.g.*, *id.*, at page 8, lines 18-36, page 39, lines 14-32.
[3] See, *e.g.*, *id.*, at page 36, lines 30-36, page 37, lines 1-2, page 16, lines 1-5, page 35, lines 25-28.
[4] See, *e.g.*, *id.*, at page 15, lines 16-28.
[5] See, *e.g.*, *id.*, at line 21.
[6] See, *e.g.*, *id.*, at line 22-23.
[7] See, *e.g.*, *id.*, at line 24.
[8] See, *e.g.*, *id.*, at line 25-26.

and R^3 is an organic acid derivative;[9]

or a salt thereof.[10]

Independent claim 98 is specifically directed to a method of killing mold in food or water comprising corn and soy.[11] Independent claim 99 specifically recites a method of killing mold in food having a moisture content of from 0-17%.[12]

VI. GROUNDS OF REJECTION TO BE REVIEWED ON APPEAL

A. Claims 75, 77-104, and 113-133 stand rejected under 35 U.S.C. § 112, second paragraph, as being vague and indefinite for failing to particularly point out and distinctly claim the subject matter that appellants regard as their invention.

B. Claims 75, 77-80, 82-87, 90-93, 96-98, 104, 113, 115-119, and 133 stand rejected under 35 U.S.C. 103(a) as obvious over Ivey *et al.* (U.S. Patent No. 5,928,686), as evidenced by Blake *et al.* (U.S. Patent No. 2,938,053), and in view of Bland *et al.* (U.S. Patent No. 5,591,467).

C. Claims 88-89 stand rejected under 35 U.S.C. 103(a) as obvious over Ivey *et al.* (U.S. Patent No. 5,928,686), as evidenced by Blake *et al.* (U.S. Patent No. 2,938,053), and in view of Bland *et al.* (U.S. Patent No. 5,591, 467) as applied to claims 75, 77-80, 82-87, 90-93, 96-98, 104, 113, 115-119, and 133, and in further view of Pinski *et al.* (U.S. Publication No. 20020172737).

D. Claims 94-95 stand rejected under 35 U.S.C. 103(a) as obvious over Ivey *et al.* (U.S. Patent No. 5,928,686), as evidenced by Blake *et al.* (U.S. Patent No. 2,938,053), and in view of Bland *et al.* (U.S. Patent No. 5,591,467) as applied to claims 75, 77-80, 82-87, 90-93,

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[9] See, *e.g.*, *Id.*, at line 27.
[10] See, *e.g.*, *id.*, at line 28.
[11] See, *e.g.*, *id.*, at page 10, lines 33-44, page 43, lines 31-34, page 44, lines 22-23.
[12] See, *e.g.*, *id.*, at page 11, lines 6-7, page 45, lines 9-31.

96-98, 104, 113, 115-119, and 133, and in further view of Friedman *et al.* (U.S. Patent No. 4,495,208).

E. Claims 114 and 120-132 stand rejected under 35 U.S.C. 103(a) as obvious over Ivey *et al.* (U.S. Patent No. 5,928,686), as evidenced by Blake *et al.* (U.S. Patent No. 2,938,053), and in view of Bland *et al.* (U.S. Patent No. 5,591,467) as applied to claims 75, 77-80, 82-87, 90-93, 96-98, 104, 113, 115-119, and 133, and in further view of Rolow *et al.* (U.S. Patent No. 6,355,289).

F. Claims 75-82 and 96 stand rejected under 35 U.S.C. 103(a) as obvious over Paquet *et al.* (CA 1261855) in view of Bland *et al.* (U.S. Patent No. 5,591,467).

G. Claims 75, 77, 97, 99-103, 113-117, and 133 stand rejected under 35 U.S.C. 103(a) in view of Doerr *et al.* (Poultry Science, 74(1), 23,1995) and Rolow *et al.* (U.S. Patent No. 6,355,289).

VII. ARGUMENT

The Examiner maintains the rejections of claims 75, 77-112, and 113-133. The arguments set forth below will address each basis of rejection under separate subheadings, in accordance with 37 C.F.R. 41.37(c)(1)(vii). Appellants will show that the rejection under 35 U.S.C. §112, second paragraph, will be rendered moot upon entry of the previously submitted amendments. Appellants will also demonstrate that a *prima facie* case of obviousness has not been established or, alternatively, that any *prima facie* case of obviousness has been rebutted. Among other considerations, it will be shown that the cited prior art has been cited out of context and does not teach or suggest each and every element recited in the claims. When each prior art reference is properly considered, it is apparent that there would have been no motivation to combine or reasonable expectation of success in combining the references in the manner cited. Importantly, the cited references include several portions that, taken as a whole, lead away from the claimed invention and contradict a finding of obviousness.[13]

It will also be shown that the methods of the claimed invention yield unexpected results, which support a finding of non-obviousness. The results of the claimed methods are substantially greater than the additive effect of what would be expected from the sum of the individual components. The results of the claimed methods are also substantially greater than any of the individual components used at a proportionally equivalent volume. Evaluation of the data of record shows that the methods of the claimed invention inhibit or kill a substantially greater number of microbial colonies in food or water than otherwise would be expected. Appellants submitted the Declaration of Dr. Knight as further evidence of unexpected results and synergistic effect. The Figure 7 identified in the Declaration shows that the claimed invention has approximately a 10-fold improvement over the prior art.[14] In view of the total evidence submitted by

[13] It is generally informative that the Office's rejections under § 103(a) rely on eight (8) different references, involving four (4) different combinations of at least three (3) references.
[14] The blends in Figure 7 are embodied by the currently claimed invention (*e.g.* claims 127 and 128 recite variations of Blend OA6).

Appellants, it is apparent that the demonstrated unexpected results and synergistic effect support a finding of non-obviousness.

Importantly, nearly every patented invention is comprised of elements that previously existed in the prior art. "However, mere identification in the prior art of each element is insufficient to defeat the patentability of the combined subject matter as a whole."[15] Previously known components may be combined and arranged in new ways that were not previously foreseen or suggested, and which are patentable. As a result, precaution must be taken to avoid hindsight bias in evaluating whether a motivation to combine and a reasonable expectation of success existed at the time of filing. For the reasons detailed below, all pending claims are not rendered obvious by any combination of references as cited by the Office.

For the purposes of this Appeal, claims 75, 77-104, and 113-133 do not stand or fall together. The claims have been divided into four groups: Group I (claims 75, 77-85, 88-104, and 133) Group II (claims 86 and 87); Group III (claims 113-118); and, Group IV (claims 119-132).

A. Claims 75, 77-104, and 113-133, as Amended, Satisfy 35 U.S.C. § 112, Second Paragraph

Claims 75, 77-104, and 113-133 were rejected under 35 U.S.C. § 112, second paragraph, as having uncertain scope because the claims recited the phrase "an acceptable diluent, adjuvant, or excipient." The claims were amended after final rejection to delete the offending phrase, and thus clarify the metes and bounds of the invention. Claim 104 was also amended after final to recite "the food" instead of "said animal food," and thus properly identify the antecedent basis. Upon entry of the identified amendments, the rejection of claims 75, 77-104, and 113-133 will be rendered moot.[16]

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[15] *In re Kahn*, 441 F.3d 977, 986 (Fed. Cir. 2006).

[16] The amendments were made to correct claim informalities and to place the rejected claims in better form for appeal. See, *e.g.*, 37 C.F.R. §1.116 (b)(2).

B. The Rejection of Claims 75, 77-80, 82-87, 90-93, 96-98, 104, 113, and 115-119 under 35 U.S.C. 103(a) over Ivey *et al.*, Blake *et al.*, and Bland *et al.* is Improper

1. The Group I Claims Under Rejection (Claims 75, 77-80, 82-85, 90-93, 96-98, and 104)

Claim 75 is representative of the Group I claims. Claim 75 is directed to a method of killing microbes in food or water. The method comprises treating the food or water with an antimicrobial composition. The antimicrobial composition comprises at least two organic acids selected from the group consisting of formic acid, butyric acid, fumaric acid, lactic acid, benzoic acid, and propionic acid; and a third organic acid that is a compound of formula (I).

The Final Action states, "Ivey *et al.* discloses a high moisture solid formulation which contains about 30% to about 90% by weight of water, and between about 10% to about 70% by weight of dry matter wherein the dry matter contains 15% by weight of 2-hydroxy-4-methylthiobutanoic acid [Alimet] . . . It is also disclosed that a high moisture having high nutrient profile is prepared by mixing soybean meal, egg white, corn starch, corn meal <u>Alimet, propionic acid</u> and citric acid."[17] (Emphasis Original). The Final Action further alleges, "Feed formulations comprising Alimet, soy oil, corn starch are also fed to the birds[18] . . . Further as evidenced by Blake *et al.* 2-hydroxy-4-(methylthio)butanoic acid has antimicrobial activity, antifungal activity, and is used in animal diet particularly poultry[19] . . . . Bland *et al.* teach that the animal feed composition for feeding animals such as poultry, swine, beef, cattle feed, dairy cattle feed, horse, aquaculture and pets comprise antibacterial agents formic acid, propionic acid, lactic acid."[20] Each of the alleged disclosures or teachings cited by the Office will be discussed in turn.

[17] See, *e.g.*, Final Office Action, mailed 12/11/2007, page 4, lines 5-11.
[18] See, *e.g.*, *id.*, at lines 12-13.
[19] See, *e.g.*, *id.*, at page 4, lines 21-22, page 5, lines 1-2.
[20] See, *e.g.*, *id.*, at page 5, lines 9-11.

a. Ivey *et al.* ("Ivey") Teaches Away; Ivey Teaches a Vehicle for Delivery of Microbes and Does Not Teach Inhibiting or Killing Microbes in Food or Water

The Office is correct that Ivey describes Alimet and propionic acid in a single composition. However, the Appellants respectfully assert that the Final Action's conclusion regarding the Ivey reference is mistaken. Ivey does not teach a method of inhibiting or killing microbes in food or water. Ivey teaches the opposite – using food as a vehicle for delivering microbes to animals.

> "The high moisture solid of the present invention, therefore, may be used as a **vehicle to administer direct-fed microbials to poultry and other animals.** When used for this purpose, the high moisture solid **should contain sufficient colony forming units of the yeast or bacterium to be of benefit to the animal.**"[21]

> "The present invention is also directed to a composition and process for **inoculating poultry and other animals with living cells such as yeast or bacteria**"[22]

As shown by the passages above, Ivey teaches that living cells such as yeast or bacteria are not inhibited or killed by Alimet and propionic acid.[23] "When a piece of prior art suggests that the line of development flowing from the reference's disclosure is unlikely to be productive of the result sought by the applicant the piece of prior art is said to **teach away** from the claimed invention."[24] Because Ivey teaches away from the currently claimed invention, there would be no motivation to use the reference for a method of inhibiting or killing microbes. Similarly, because Ivey teaches a vehicle for delivering microbes in food, there would be no expectation of success for using its constituents for a method of inhibiting or killing microbes in food or water.

[21] See, *e.g.*, Ivey *et al.* at col. 6, lines 8-19. (Emphasis Added).
[22] See, *e.g.*, *id.* at col. 2, lines 65-67. (Emphasis Added).
[23] See, *e.g.*, *id.* at claims 13, 14, 36, and 37.
[24] *In re Gurley*, 27 F.3d 551, 553 (Fed. Cir. 1994). (Emphasis Added).

b. Ivey Rebuts Prima Facie Obviousness of Appellants' Claimed Invention by Showing Unexpected Results

As shown above, Ivey discloses both Alimet and propionic acid for a composition that is used to deliver microbials in food.[25] Ivey is evidence of unexpected results because one of skill in the art would not expect that ingredients of a composition used for delivering microbes in food could be combined with a third ingredient to provide a contrary method for inhibiting or killing microbes in food or water. As provided by the Manual of Patent Examining Procedure (MPEP), "Presence of a property not possessed by the prior art is evidence of non-obviousness."[26] At a minimum, Appellants have rebutted the Office's inaccurate contention that "the methods as taught by Ivey . . . *necessarily result in killing microbes in food*, as recited in the claims."[27]

c. Ivey Does Not Teach or Suggest All Elements of Appellants' Claimed Invention

The claims currently on appeal are all directed to methods for killing microbes in feed or water that comprise at least two organic acids selected from the group consisting of formic acid, butyric acid, fumaric acid, lactic acid, benzoic acid, and propionic acid; and a compound of formula (I). In addition to Ivey failing to teach or suggest a method for killing microbes in food or water, Ivey also does not teach the at least two organic acids as claimed by Appellants. Ivey does not teach a compound of formula (I) to be useful as an antimicrobial.[28] Considering that the alleged "antimicrobial" composition of Ivey was actually a vehicle for delivering microbials, it is apparent that there is no motivation to combine or reasonable expectation of success regarding the cited art. In addition to the noted teachings away, Ivey further suggests that there is substantial unpredictability regarding so-called "antimicrobials" in the chemical

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[25] See, *e.g.*, Ivey *et al.* at col. 5, lines 49-67, col. 6, lines 1-7.
[26] See, *e.g.*, MPEP § 716.02(a); *In re Papesch*, 315 F.2d 381 (CCPA 1963).
[27] See, *e.g.*, Final Action at page 6, lines 16-18. (Emphasis Added).
[28] See, *e.g.*, Ivey at col. 4, lines 59-67, col. 5, lines 1-14.

arts. As stated by MPEP § 2143.02, "[A]t least some degree of predictability is required. Evidence showing there was no reasonable expectation of success may support a conclusion of non-obviousness."[29]

d. Blake *et al.* ("Blake") Teaches Away; Blake Describes Methionine Analogues Not Useful as Animal Feed Supplements

The Final Action's reliance on the Blake patent is also misplaced. The passages cited and relied on by the Office (*i.e.*, Blake *et al.*, at col. 1, lines 39-41) does not actually refer to 2-hydroxy-4-(methylthio)butanoic acid but to new chemical variants with substantially different molecular structure and function.

> "Other methionine analogues **differ considerably from the natural methionine in molecular structure** and because of the unnatural configuration are **not useful as animal feed supplements**. Many of these are absorbed by the plant and animal structures and **have toxic effects** due to the inability of the organism to assimilate the analogue . . . Thus many of the new compounds are useful as fungicides, bactericides, virus control agents [etc.]"[30]

Even though the new compounds are deadly to microorganisms, the Blake patent **teaches away** from use of these variants in animal food and water by reciting they are "**not useful as animal feed supplements**" and "**have toxic effects**." (Emphasis Added). Even if these toxic chemicals could theoretically be regulated as applied to the surface of plants or animals to remove certain microorganisms, the Blake patent **provides no teachings for how these variant chemicals could be ingested or combined with food supplements**. Therefore, these toxic analogues are considerably different from the previously known animal feed additives and methionine derivatives. Blake fails to provide the necessary teachings as relied upon by the Final Action, and teaches away since the new compounds are not useful for animal feed supplements.

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[29] See, *e.g.*, MPEP § 2143.02; *In re Rinehart*, 531 F.2d 1048 (CCPA 1976).
[30] See, *e.g.*, Blake *et al.* at col. 1, lines 31-42. (Emphasis Added).

e. Bland *et al.* ("Bland") Teaches Away; Organic Acids Are Not Effective At Killing Bacteria in Foodstuffs Without Formaldehyde

The Office relies on Bland to support "that the animal feed composition[s] . . . comprise antibacterial agents formic acid, propionic acid, lactic acid."[31] Although the cited animal feed compositions do include these ingredients, Bland *et al.* states that the ingredients are not effective at killing bacteria in animal feedstuffs, including *Salmonella*.

> "**[M]any compounds with known bacteriocidal properties**, such as lactic acid, propionic acid, formic acid, butyric acid, sorbic acid, benzoic acid and combinations of these have been tested. While many of these agents kill bacteria in solution, **they do not kill all the bacteria in animal feedstuffs**. Woolford, M. K., "Microbiological Screening of Food Preservatives, Cold Sterilants and Specific Antimicrobial Agents as Potential Silage Additives", J. Sci. Ed. Agric. 1975, 26, 229-237. **To be effective against Salmonella, a bacteriocidal treatment must kill essentially all of the bacteria**. Methods that kill 95% or even 99% are largely ineffective because the residual bacteria can multiply rapidly and recontaminate the feedstuff, and eventually the entire processing facility."[32]

"A prior art reference must be considered in its entirety, *i.e.*, as a whole, including portions that would lead away from the claimed invention."[33] Taken in context, **Bland actually teaches that the required key ingredient for a bactericidal composition is formaldehyde**. (Emphasis Added).

> "[S]uch **treatments fail to eliminate the Salmonella effectively when too little formaldehyde is used** or when the solution is not sprayed uniformly onto the

[31] See, *e.g.*, Final Action at page 5, lines 9-11.
[32] See, *e.g.*, Bland *et al.* at col. 2, lines 20-34. (Emphasis Added).
[33] See, e.g., MPEP § 2141.02; *W.L. Gore & Associates, Inc. v. Garlock, Inc.*, 721 F.2d 1540 (Fed. Cir. 1983).

> feedstuff, thereby allowing some small number of bacteria to survive and multiply."[34]

Because one of skill in the art would view all embodiments of the Bland patent to expressly or implicitly require formaldehyde in order to successfully inhibit or kill microbes in food compositions, the Bland reference may be said to teach away. The currently claimed invention does not recite or require any formaldehyde, and formaldehyde fails to qualify as a compound of formula (I). Consequently, Bland fails to teach a compound of formula (I) and further provides no expectation of success for using organic acids without the addition of large amounts of formaldehyde.

f. *In re Kerkhoven* Does Not Properly Apply

Due to the contradictory teachings of the prior art as indicated above, the Office's reliance on *In re Kerkhoven*, 626 F.2d 848 (CCPA 1980) in the Final Action is misplaced. *In re Kerkhoven* was cited on the belief that the cited references show antimicrobial agents that are useful for the same purpose. However, close evaluation of cited prior art has revealed that these teachings do not exist or, alternatively, that the individual components are ineffective or insufficient for the purposes of the currently claimed invention. In a number of instances, it has been shown that the prior art actually teaches away from the claimed methods. Ivey does not teach a method of inhibiting or killing microbes, but rather the opposite: a microbial vehicle for delivering microbes in food. While Blake describes variants of methionine with toxic effects, these particular chemicals are recited as unacceptable for animal feed. Finally, Bland teaches away by reciting that several organic acids are bacteriocidal in solution, but are insufficient at killing bacteria in feedstuffs without large amounts of formaldehyde. As such, the currently claimed invention is not taught or suggested by the prior art, and *In re Kerkhoven* does not properly apply.

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[34] See, *e.g.*, Bland *et al.* at col. 2, lines 39-43. (Emphasis Added).

g. There Is No Motivation to Combine Ivey, Blake, and Bland

One of skill in the art would not have been motivated to combine the references as cited by the Office in order make the claimed invention. Nowhere in the prior art have the claimed methods of using such combinations of compounds been taught or suggested. As shown previously, one of skill in the art could not rely on any inherent properties of the claimed compounds since there was a variety of teachings away and unpredictability in the field. None of the prior art cited by the Final Action teaches or suggests a method of inhibiting or killing microbes using a compound a formula (I) in combination with at least two organic acids selected from formic acid, butyric acid, fumaric acid, lactic acid, benzoic acid, and propionic acid.

Even though each of the cited references has one or more components of the claimed invention, when each reference is considered in its entirety, it has been demonstrated that the prior art teaches away from such a combination.

> "[M]ere identification in the prior art of each component of a composition does not show that the combination as a whole lacks the necessary attributes for patentability. Rather, to establish a prima facie case of obviousness based on a combination of elements in the prior art, **the law requires a motivation to select the references and to combine them in the particular claimed manner to reach the claimed invention**."[35]

The below passage of Ivey further shows that there may be an infinite number of food additives that could potentially be used to modify the microbial characteristics of a food composition.

> "The present invention is further directed to a high moisture solid for improving the health, livability, cumulative weight gain or feed conversion efficiency

[35] *Eli Lilly & Co. v. Zenith Goldline Pharms.*, Inc., 471 F.3d 1369, 1379 (Fed. Cir. 2006) (internal citations omitted). (Emphasis Added).

> of poultry. The high moisture solid may comprise, for example, between about 50% and about 95% by weight water, between about 5% and about 50% by weight dry matter, **and an additive** selected from the group consisting of **bile salts, surfactants, enzymes, enzyme co-factors, hormones, prostaglandins, peptides, immunoglobulins, cytokines, antioxidants, amino acids and sources of amino acids and amino acid analogs, antibiotics, vitamins and minerals.**"[36]

There is no indication, however, which additives or combination of additives may be successful at inhibiting or killing microbes in food or water. This is particularly true since Ivey has shown that some microorganisms may grow and thrive in acidic environments, including those containing Alimet and propionic acid.

h. There Is No Expectation of Success In Combining Ivey, Blake, and Bland

Even if the Ivey, Blake, and Bland references were combined, the prior art has shown that there would be no expectation of success for inhibiting or killing microbes in food or water. For example, the Ivey reference teaches a vehicle for delivering microbes, rather than a composition for inhibiting or killing them. Likewise, the chemicals described in the Blake *et al.* reference were recited to have toxic effects and unacceptable as animal food supplements. There is also the Appellants' previously submitted evidence.[37]

The Declaration of Dr. Knight under 37 C.F.R. §1.132 shows that the individual organic acids are inadequate for the limitations of the claimed invention. The following passage from the Declaration states that the methods of the claimed invention also demonstrate unexpected results.

> "... [w]e have research data, that in my opinion, **demonstrates surprising and unexpected results for organic acid formulations falling within the scope of the '434 patent claims**. As an example,

[36] See, e.g., Ivey *et al.* at col. 3, lines 6-14. (Emphasis Added).
[37] A copy of Dr. Knight's declaration was submitted to the USPTO on September 26, 2007, as part of the response to the non-final Office Action mailed March 27, 2007.

> attached to this Declaration is a graph (identified as figure 7) that depicts a synergistic effect. . . ."[38]

As such, the Declaration is evidence that further supports allowance of the presently pending claims.

In addition to the Declaration of Dr. Knight, the Appellants also previously submitted the Warnecke *et al.* review article as part of the response to the non-final Office Action mailed March 27, 2007.[39] As evidenced by the review article, the state of knowledge in the microbial arts at the time of filing supports the notion that a random selection of organic acids would be unpredictable for the purpose of the currently claimed invention. The Warnecke *et al.* review article exemplifies this unpredictability, and reveals that many microorganisms may live and thrive in acidic environments.[40] Individual organic acids uniquely, and at times unpredictably, impact microbe cell growth, regulatory pathway, turgor pressure, and cell landscape.[41] Every organic acid may potentially cause a unique response by an individual microorganism. Additionally, the degree of bioavailability (*i.e.*, ability to reach the target microbe) varies for different organic

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[38] 37 C.F.R 1.132 Declaration of Dr. Christopher Knight, at paragraph 4, a copy of which was submitted with the response to the Office Action dated March 27, 2007. (Emphasis Added).
[39] A copy of the Warnecke *et al.* review article was submitted to the USPTO on September 26, 2007, as part of the response to the non-final Office Action mailed March 27, 2007.
[40] Warnecke, T., and Gill, R., Microbial Cell Factories (2005) 4:25, a copy of which was submitted with the response to the Office Action dated March 27, 2007.
[41] *Id.* For example, see the third page, column two of the article, which states:
Organic acid anions affect cell growth in a variety of manners. Increased anion concentration has been shown to lead to an increased transport of potassium ions into the cell, which increases turgor pressure [47,48]. To maintain a constant turgor pressure and cell volume, glutamate is transported out of the cell [48]. This transport activity concomitantly disrupts the osmolarity of the cytoplasm, which in turn lowers the cell's growth potential and viability. In addition to this general anion effect, **there are also effects specific to each organic acid.** It has been proposed that enzymes involved in protein synthesis are sensitive to a combination of two unrelated mechanisms, including the acidification of pHi and the formation of an anionic pool [35]. Although this finding implies that the **organic inhibition due to the anion pool could be acid specific**, the details describing this dual inhibition mechanism remain unclear. Kirkpatrick et al. reported proteins exhibiting increased expression in response to extracellular acetate [33]. Among these are the OppA transporter, RpoS regulon, several amino acid uptake proteins, DNA binding proteins, and extreme-acid preiplasmic chaperones. Interestingly, when formate was introduced in place of acetate the expression of the previously mentioned proteins was repressed, **indicating that the response was anion specific**. This finding introduces new challenges in addressing organic acid tolerance. Specifically, it highlights the need to engineer both pH and as well as specific anion tolerance into host organisms. (Emphasis added).

acids, and different microbes are resistant to different pH ranges. With this degree of unpredictability, a skilled artisan empowered with the cited prior art and the general knowledge of the microbial arts would not have a reasonable expectation of success in combining references as indicated by the Final Action.

i. Previously Submitted Evidence Supports a Finding of Non-Obviousness

"Evidence rebutting a *prima face* case of obviousness can include: 'evidence of unexpected results,' [and] evidence 'that the prior art teaches away from the claimed invention in any material respect' . . . When a patent applicant puts forth rebuttal evidence, the Board must consider that evidence."[42] In the present case, the Appellants have previously submitted substantial evidence of unexpected results to rebut a finding of obviousness. While synergism is not a requirement of non-obviousness,[43] it has been shown that synergism and unexpected results exist in the present case. The combination of the claimed invention is greater than the additive effect of what would be expected from the sum of the individual components. When synergism is present, particularly in a chemical case, it is indicative of non-obviousness.[44]

The data of record shows that the combination of the claimed invention kills a substantially greater number of microbial colonies, as compared to the organic acids tested. For example, in Figure 7 that accompanied the Declaration of Dr. Knight, Blend OA 4 and Blend OA 6 were shown to have approximately a 10-fold improvement compared to any of the single organic acid compositions tested at equivalent volumes. Blends OA 4 and OA 6 are embodied by the currently claimed invention. By way of example, claims 127 and 128 specifically recite the composition of Blend OA6.

While the Office previously asserted that the results submitted via Dr. Knight's Declaration were "not convincing" because no data was shown for

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[42] See, *e.g.*, *In re Sullivan*, 498 F.3d 1345, 1351 (Fed. Cir. 2007) (internal citations omitted).
[43] *Gardner v. TEC Sys. Inc.*, 725 F.2d 1338, 1349 (Fed. Cir. 1984) (en banc).
[44] *Stratoflex, Inc. v. Aeroquip Corp.*, 713 F.2d 1530, 1540 (Fed. Cir. 1983).

propionic acid alone, this assertion is misplaced. The previously cited Ivey reference provides evidence that propionic acid in combination Alimet does not reliably inhibit or kill microbes in food or water. Similarly, the previously cited Bland reference states that ". . . many compounds with known bacteriocidal properties, **such as . . . propionic acid . . . and combinations of these** have been tested. While many of these agents kill bacteria in solution, **they do not kill all the bacteria in animal feedstuffs**."[45] Therefore, the general state of the art supports the non-obviousness evidence submitted by the Appellants. Finally, the evidence is further supported by the previously submitted comparison between propionic acid and HMTBA, which indicated propionic acid alone is ineffective for the purposes of the claimed invention.[46] As such, the evidence of record plainly shows that the currently claimed invention is, as a whole, non-obvious.

Accordingly, the rejection of claims 75, 77-80, 82-85, 90-93, 96-98, and 104 under 35 U.S.C. 103(a) over Ivey, Blake, and Bland is improper.

2. The Group II Claims Under Rejection (Claims 86 and 87)

Claim 86 is representative of the Group II claims under rejection. Claim 86 is directed to a method of killing microbes in food or water. The method comprises treating the food or water with an antimicrobial composition, and feeding to **a ruminant animal**. (Emphasis Added). The antimicrobial composition fed to the ruminant animal comprises at least two organic acids selected from the group consisting of formic acid, butyric acid, fumaric acid, lactic

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[45] See, *e.g.*, Bland *et al.* at col. 2, lines 20-34. (Emphasis Added).

[46] The comparison between propionic acid and HMTBA for Salmonella was previously submitted by the Appellants and entered into the record pursuant to 37 C.F.R. §1.116(e). The Advisory Action mailed May 14, 2008 indicated that the request for reconsideration had been considered and made of record, even though the specifically requested claim amendments were not permitted. Furthermore, the Appellants had good and sufficient reasons why the affidavit was necessary and was not earlier presented, since the Examiner had specifically stated that "HMTBA is not convincing because no data is provided for the propionic acid alone for comparison." See, *e.g.*, Final Action at page 16, lines 22-23. As such, the recited evidence was properly entered into the record.

acid, benzoic acid, and propionic acid; and a third organic acid that is a compound of formula (I).

The arguments asserted above in Part B(1) are hereby reasserted with respect to Part B(2) and claims 86 and 87. In particular, Ivey, Blake, and Bland have been previously shown above to teach away from the currently claimed invention, provide no motivation to combine, and give no expectation of success regarding inhibiting or killing microbes in food or water. Ivey describes a vehicle for delivering microbes whereas the compositions of Blake are "not useful for animal food supplements" and have "toxic effects." More specifically, the cited art combination provides no motivation to combine and no expectation of success regarding **ruminant animals.** (Emphasis Added). The Final Action specifically states that "**Ivey do not specifically teach that the formulation are mixed with food for feeding ruminant animal.**"[47] Similarly, Blake provides no teachings regarding ruminant animals.

Bland does not cure the failings of Ivey or Blake. As shown above, Bland specifically recites that organic acids are not effective at killing microbes in animal feedstuffs without large amounts of formaldehyde.

> "**[M]any compounds with known bacteriocidal properties**, such as lactic acid, propionic acid, formic acid, butyric acid, sorbic acid, benzoic acid and combinations of these have been tested. While many of these agents kill bacteria in solution, **they do not kill all the bacteria in animal feedstuffs**. . . ."[48]

This teaching away by Bland contradicts any supposed motivation to combine the references cited in this rejection, including with respect to feeding ruminant animals. Finally, Bland does not provide any teachings regarding a compound of formula (I), as required by the Group II claims.

Accordingly, the rejection of claims 86 and 87 under 35 U.S.C. 103(a) over Ivey, Blake, and Bland is improper.

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[47] See, *e.g.*, Final Action at page 5, lines 7-8. (Emphasis Added).
[48] See, *e.g.*, Bland *et al.* at col. 2, lines 20-34. (Emphasis Added).

3. The Group III Claims Under Rejection (Claims 113 and 115-118)

Claim 113 is representative of the Group III claims under rejection. Claim 113 is directed to a method of killing microbes in food or water. The method comprises treating the food or water with an antimicrobial composition. The antimicrobial composition comprises at least two organic acids selected from the group consisting of formic acid, butyric acid, fumaric acid, lactic acid, benzoic acid, and propionic acid; and a third organic acid that is a compound of formula (I), **wherein the compound of formula (I) is 2-hydroxy-4-(methylthio)butanoic acid**. (Emphasis Added).

The arguments asserted above in Parts B(1) and B(2) are hereby reasserted with respect to Part B(3) and claims 113 and 115-118. In particular, Ivey, Blake, and Bland have been previously shown to teach away from the currently claimed invention, provide no motivation to combine, and give no expectation of success regarding inhibiting or killing microbes in food or water. The cited art combination provides no motivation to combine and no expectation of success regarding **2-hydroxy-4-(methylthio)butanoic acid as a compound of formula (I)** for a method of inhibiting or killing microbes in food or water. (Emphasis Added).

As shown above, Ivey teaches away by describing a vehicle for delivering microbes. Further, Ivey uses Alimet as a protein supplement and provides no teachings for its use as an antimicrobial. Blake does not provide any teachings regarding **2-hydroxy-4-(methylthio)butanoic acid, but only to variants that have toxic effects and are unacceptable for animal feed supplements.** Bland recites that organic acids are not effective at killing microbes in animal feedstuffs without large amounts formaldehyde, and provides no teachings regarding a compound of formula (I). As such, one of skill in the art would have no motivation to combine or expectation of success regarding a combination of at least two organic acids and a compound of formula (I). More specifically, one of skill in the art would also no motivation to combine or expectation of success

regarding 2-hydroxy-4-(methylthio)butanoic acid, an element specifically required by the Group III claims.[49]

Accordingly, the rejection of claims 113 and 115-118 under 35 U.S.C. 103(a) over Ivey, Blake, and Bland is improper.

4. The Group IV Claims Under Rejection (Claim 119)

Claim 119 is representative of the Group IV claims under rejection. Claim 119 is directed to a method of killing microbes in food or water. The method comprises treating the food or water with an antimicrobial composition. The antimicrobial composition comprises 2-hydroxy-4-(methylthio)butanoic acid, formic acid, and propionic acid, wherein **the content of 2-hydroxy-4-(methylthio)butanoic acid is from about 5% to about 20% of the sum of the 2-hydroxy-4-(methylthio)butanoic acid, formic acid, and propionic acid content; the content of the formic acid is from about 65% to about 85% of said sum; and the content of the propionic acid is from about 1% to about 15% of said sum.** (Emphasis Added).

The arguments asserted above in Parts B(1)-(3) are hereby reasserted with respect to Part B(4) and claim 119. In particular, Ivey, Blake, and Bland have been previously shown to teach away from the currently claimed invention, provide no motivation to combine, and give no expectation of success regarding inhibiting or killing microbes in food or water. Even more specifically, the cited art combination provides no motivation to combine and no expectation of success regarding **specific percentages** of 2-hydroxy-4-(methylthio)butanoic acid, formic acid, and propionic acid **as claimed by claim 119.** (Emphasis Added). The teachings away as identified above and the unpredictability in the microbial arts indicate that the claimed percentages would not have been within the skill in the art. As such, the Office's reliance on *In re Bosch*, 205 USPQ 215 (CCPA 1980)

[49] See, *e.g.*, Bland *et al.* at col. 2, lines 20-34. (Emphasis Added).

for the selection of "optimal parameters"[50] is not supported by either the cited art or the general state of the technology.

Accordingly, the rejection of claim 119 under 35 U.S.C. 103(a) over Ivey, Blake, and Bland is improper.

C. The Rejection of Claims 88-89 under 35 U.S.C. 103(a) over Ivey, Blake, Bland, and Pinski *et al.* ("Pinski") is Improper

Claims 88-89 are directed to methods of killing microbes in food or water fed to an aquaculture animal, and belong to the Group I claims. Claim 75 is representative of the Group I claims. Claim 75 is directed to a method of killing microbes in food or water. The method comprises treating the food or water with an antimicrobial composition. The antimicrobial composition comprises at least two organic acids selected from the group consisting of formic acid, butyric acid, fumaric acid, lactic acid, benzoic acid, and propionic acid; and a third organic acid that is a compound of formula (I).

The arguments asserted above in Part B are hereby reasserted with respect to Part C and claims 88-89. In particular, Ivey, Blake, and Bland have been previously shown to teach away from the currently claimed invention, provide no motivation to combine, and give no expectation of success regarding inhibiting or killing microbes in food or water. The Office has cited Pinksi because it generally relates to aquaculture and is said to disclose antimicrobial agents "selected from propionic acid, salt of propionic acid, citric acid, or a salt thereof."[51]

Importantly Pinksi does not teach, disclose, or suggest any compound of formula (I) as required by the currently claimed invention. Moreover, Pinski cannot be properly combined with Ivey or Blake because these references have been shown to teach away from antimicrobials and animal feeds, respectively. Ivey describes a vehicle for delivering microbes and whereas the compositions of Blake are "not useful for animal food supplements" and have "toxic effects."

[50] See, *e.g.*, Final Action at page 7, lines 7-9. (Emphasis Added).
[51] See, e.g., Final Action at page 8, lines 1-2.

In fact, the teachings of Pinski are limited to oil-coated, encapsulated, moistured aquaculture feed having a particle size of less than about 1000 micrometers. Pinksi provides no teachings for foods that are not oil-coated and encapsulated. Pinski also teaches away from the claimed invention by packaging foodstuff with bacteria that do not appear to be adversely effected, inhibited, or killed by the so-called antimicrobials.[52]

> "In one aspect, powdered feed, endo-probiotic bacteria and/or ecto-probiotic bacteria, water and oil are mixed to provide a feed which not only can enhance the value of the feed for certain species of aquatic life, such as shrimp, but the release of such bacteria can help maintain a clean water environment . . . **<u>Endo-probiotic bacteria which may be used in the product of the invention include dried B. licheniformis and B. subtilis strains commercially available</u>** . . ."[53]

It has also been shown by Bland that the specific organic acids listed by Pinski[54] are not effective at killing microbes in animal feedstuffs without large amounts of formaldehyde. This teaching away by Bland contradicts any supposed motivation to combine the references cited in this rejection, and supports a finding of non-obviousness.

Accordingly, the rejection of claims 88-89 under 35 U.S.C. 103(a) over Ivey, Blake, Bland, and Pinski is improper.

D. The Rejection of Claims 94-95 under 35 U.S.C. 103(a) over Ivey, Blake, Bland, and Friedman *et al.* ("Friedman") is Improper

Claims 94-95 are directed to a method of killing microbes in food or water that is fed to a companion animal, and belong to the Group I claims. Claim 75 is representative of the Group I claims. Claim 75 is directed to a method of killing microbes in food or water. The method comprises treating the food or water with

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[52] See, *e.g.*, Pinski *et al.* at published paragraph [0015].
[53] See, *e.g.*, *id.* (Emphasis Added)
[54] See, *e.g.*, *id.* at published paragraph [0010].

an antimicrobial composition. The antimicrobial composition comprises at least two organic acids selected from the group consisting of formic acid, butyric acid, fumaric acid, lactic acid, benzoic acid, and propionic acid; and a third organic acid that is a compound of formula (I).

The arguments asserted above in Parts B and C are hereby reasserted with respect to Part D and claims 94-95. The Office cites Friedman because it is alleged that it teaches "pet food for feeding pets such as dog food contains antibacterial agents."[55]

Ivey, Blake, and Bland have been previously shown to teach away from the currently claimed invention, provide no motivation to combine, and give no expectation of success regarding inhibiting or killing microbes in food or water. Friedman does not teach, disclose, or suggest any compound of formula (I) as claimed by the Appellants. Moreover, Friedman cannot be combined with Ivey or Blake because these references have been shown to teach away from antimicrobials and animal feeds, respectively. It has also been shown by Bland that the organic acids disclosed by Friedman[56] are not effective at killing microbes in animal feedstuffs without large amounts of formaldehyde. This teaching away by Bland contradicts any supposed motivation to combine or expectation of success, and supports a finding of non-obviousness.

Accordingly, the rejection of claims 94-95 under 35 U.S.C. 103(a) over Ivey, Blake, Bland, and Friedman is improper

E. The Rejection of Claims 114 and 120-132 under 35 U.S.C. 103(a) over Ivey, Blake, Bland, and Rolow *et al.* ("Rolow") is Improper

1. The Group III Claims Under Rejection (Claim 114)

Claim 114 is representative of the Group III claims under rejection. Claim 114 is directed to a method of killing microbes in food or water. The method comprises treating the food or water with an antimicrobial composition. The

[55] See, *e.g.*, Final Action at page 8, lines 20-21.
[56] See, *e.g.*, Friedman et al. at col. 3, lines 64-67, col. 4, lines 1-16.

antimicrobial composition comprises at least two organic acids selected from the group consisting of formic acid, butyric acid, fumaric acid, lactic acid, benzoic acid, and propionic acid; and a third organic acid that is a compound of formula (I), **wherein the compound of formula (I) is 2-hydroxy-4-(methylthio)butanoic acid**. The method of claim 114 further comprises **an acidulant selected from the group consisting of phosphoric acid, sulfuric acid, phosphorous acid, hydrochloric acid, hydrobromic acid, and nitric acid.** (Emphasis Added).

The arguments asserted above in Parts B-D are hereby reasserted with respect to Part E and claim 114. The Office cites the Rolow reference as allegedly disclosing a liquid preservation composition to extend the shelf life of tortillas made from corn. In a preferred embodiment, the preservation composition of Rolow is said to comprise "phosphoric acid, propionic acid, and benzoic acid."[57]

Ivey, Blake, and Bland have been previously shown to teach away from the currently claimed invention, provide no motivation to combine, and give no expectation of success regarding inhibiting or killing microbes in food or water. Rolow does not teach, disclose, or suggest any compound of formula (I) as claimed by the Appellants. Even more specifically, **Rolow *et al.* fails to teach 2-hydroxy-4-(methylthio)butanoic acid as required by claim 114**. (Emphasis Added). Rolow cannot be combined with Ivey or Blake because these references have been shown to teach away from antimicrobials and animal feeds, respectively. It has also been shown by Bland that the organic acids disclosed by Rolow are not effective at killing microbes in animal feedstuffs without large amounts of formaldehyde. This teaching away by Bland contradicts any supposed motivation to combine or expectation of success, and supports a finding of non-obviousness.

[57] See, *e.g.*, Final Action at page 9, lines 18-21.

More specifically, Rolow is limited to tortillas and products made from tortilla flour.[58, 59] Rolow indicates that a number of known antimicrobial preservatives, including those claimed by Appellants, are unacceptable for individual use in tortillas because they adversely affect taste and odor.[60] **Rolow specifically identifies fumaric acid and benzoic acid to be unsatisfactory as individual antimicrobial agents in tortillas, giving an off flavor and being ineffective at controlling growth of high level organisms**.[61, 62] (Emphasis Added). Fumaric acid and benzoic acid are two of the organic acids specifically recited in the Group III claims. The teachings away by Rolow may not be disregarded, since two of the "primary indications of spoilage in tortillas is an off odor or taste . . ."[63] Taken in context, it is apparent that only the specific combination of acids described by Rolow actually yields a "surprisingly . . . fresh taste with a slight sweetness at the finish" for tortillas.[64, 65] One of skill in the art would therefore view Rolow as describing an **unusual combination that acts contrary to the prior art, contrary to the individual properties of the**

[58] "[I]t can be seen that the combination of benzoic acid with propionic acid and phosphoric acid, in the proportions specified, is an effective preservative for products made from tortilla flour." (See, e.g., Rolow *et al.* at col.7, lines 47-51).
[59] "This invention relates generally to methods and chemicals for extending the shelf life of corn tortillas or wheat tortillas, and specifically the preservation of corn tortillas or wheat tortillas . . ." (See, *e.g.*, *id.* at col. 1, lines 12-15).
[60] "Various antimicrobial preservatives have been proposed, however they have limitations of increasing the cost of producing tortilla and/or adversely affecting the taste and odor." (See, *e.g.*, *id.* at col. 1, lines 35-38).
[61] "[A]cidulants such as fumaric acid or citric acid, are used to reduce pH levels. A major drawback resulting from this type of preservative mixture is the lingering after-taste of the acidulant. These preservative mixtures have successfully increased the shelf life of tortillas . . . However, the taste of the tortillas containing these preservatives has not been satisfactory. Also the supply of some of these preservatives have been limited, making them difficult to or expensive to obtain." (See, *e.g.*, *id.* at col. 2, lines 5-13).
[62] "Benzoic acid is a well-known food preservative. . . generally used only in very acidic foods such as pickles, soft drinks and dressings. . . Benzoic acid is also known to impart an off flavor. Because of the narrow pH range in which it has generally been effective and because of its off-flavor, it is being replaced by other preservatives. Benzoic acid has not been effective to control the growth of high-levels of microorganisms. Because tortillas generally have a pH level above the optimum effective antimicrobial range of benzoic acid, benzoic acid has not been commonly used as a tortilla preservative." (See, *e.g.*, *id.* at col. 3, lines 12-27) (internal citations omitted).
[63] See, *e.g.*, *id.* at col. 1, lines 35-38.
[64] See, *e.g.*, *id.* at col. 4, lines 16-19.
[65] See, *e.g.*, *id.* at col. 4, lines 43-46.

ingredients, and a combination that is likely limited to tortilla products.[66] At a minimum, it is entirely unclear whether the addition of another chemical, such as a compound of formula (I), would disturb the unusual properties of the Rolow antimicrobial composition. Rolow provides no teachings whatsoever regarding a compound of formula (I), or whether it would be compatible with the combination described therein. This fact is even acknowledged by the Office at page 9 of the Final Action at lines 16-17, "**The combination of references do not specifically teach the employment of butyric acid, phosphoric acid, and the particular amounts of said acids**." (Emphasis Added).

Accordingly, the rejection of claim 114 under 35 U.S.C. 103(a) over Ivey, Blake, Bland, and Rolow is improper.

2. The Group IV Claims Under Rejection (Claims 120-132)

Claim 120 is representative of the Group IV claims under rejection. Claim 120 is directed to a method of inhibiting killing microbes in food or water. The method comprises treating the food or water with an antimicrobial composition. The antimicrobial composition comprises at least two organic acids selected from the group consisting of formic acid, butyric acid, fumaric acid, lactic acid, benzoic acid, and propionic acid; and a third organic acid that is a compound of formula (I). More specifically, **claim 120 requires the 2-hydroxy-4-(methylthio)butanoic acid to be from about 5% to about 20% of the sum of the 2-hydroxy-4-(methylthio)butanoic acid, formic acid, and propionic acid content; the content of the formic acid is from about 65% to about 85% of said sum; and the content of the propionic acid is from about 1% to about 15% of said sum. Claim 120 further comprises phosphoric acid, wherein the content of the phosphoric acid is from about 5% to about 20% of said sum**. (Emphasis Added).

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[66] *Id.*

The arguments asserted above in Parts B-E(1) are hereby reasserted with respect to Part E(2) and claims 120-132. In particular, Ivey, Blake, Bland, and Rolow have been previously shown to teach away from the currently claimed invention, provide no motivation to combine, and give no expectation of success regarding inhibiting or killing microbes in food or water. Even more specifically, the cited art combination provides no motivation to combine and no expectation of success regarding **specific the percentages** of 2-hydroxy-4-(methylthio)butanoic acid, formic acid, and propionic acid **as recited by claim 120.** (Emphasis Added). Resort to Bland does not cure the failings of Ivey, Blake, or Rolow because, as shown above.[67] Bland does not provide any teachings regarding 2-hydroxy-4-(methylthio)butanoic acid, as specifically required by the Group IV claims. Rolow also fails to provide any teachings regarding 2-hydroxy-4-(methylthio)butanoic acid. Overall, these failings of the prior art are acknowledged by the Office at page 9 of the Final Action at lines 16-17, which states, "**The combination of references do not specifically teach the employment of butyric acid, phosphoric acid, and the particular amounts of said acids**." (Emphasis Added).

Accordingly, the rejection of claims 120-132 under 35 U.S.C. 103(a) over Ivey, Blake, Bland, and Rolow is improper.

F. The Rejection of Claims 75-82 and 96 under 35 U.S.C. 103(a) over Paquet *et al.* ("Paquet") and Bland is Improper

Claims 75-82 and 96 are directed to methods of killing microbes in food or water, and belong to Group I. Claim 75 is representative of the Group I claims. Claim 75 is directed to a method of inhibiting killing microbes in food or water. The method comprises treating the food or water with an antimicrobial composition. The antimicrobial composition comprises at least two organic acids selected from the group consisting of formic acid, butyric acid, fumaric acid, lactic acid, benzoic acid, and propionic acid; and a third organic acid that is a

[67] See, *e.g.*, Bland *et al.* at col. 2, lines 20-34. (Emphasis Added).

compound of formula (I). The arguments asserted above in Parts B-E, particularly including the subsections relating to the Bland reference, are hereby reasserted with respect to Part F and claims 75-82 and 96.

Bland Teaches Away; Organic Acids Are Not Effective At Killing Bacteria in Foodstuffs Without Formaldehyde

To reiterate, Bland states that organic acids such as propionic acid are not effective at killing bacteria in animal feedstuffs.

> "**[M]any compounds with known bacteriocidal properties**, such as lactic acid, propionic acid, formic acid, butyric acid, sorbic acid, benzoic acid and combinations of these have been tested. While many of these agents kill bacteria in solution, **they do not kill all the bacteria in animal feedstuffs**. Woolford, M. K., "Microbiological Screening of Food Preservatives, Cold Sterilants and Specific Antimicrobial Agents as Potential Silage Additives", J. Sci. Ed. Agric. 1975, 26, 229-237. **To be effective against Salmonella, a bacteriocidal treatment must kill essentially all of the bacteria**. Methods that kill 95% or even 99% are largely ineffective because the residual bacteria can multiply rapidly and recontaminate the feedstuff, and eventually the entire processing facility."[68]

Bland further teaches that the large amounts of formaldehyde are required to eliminate microbes such as Salmonella.

> "[S]uch **treatments fail to eliminate the Salmonella effectively when too little formaldehyde is used** or when the solution is not sprayed uniformly onto the feedstuff, thereby allowing some small number of bacteria to survive and multiply."[69]

Because Bland expressly or implicitly requires formaldehyde in order to successfully inhibit or kill microbes in food compositions, the Bland reference

[68] See, *e.g.*, Bland *et al.* at col. 2, lines 20-34.
[69] See, *e.g.*, *id.* at col. 2, lines 39-43.

may be said to teach away from the currently claimed invention. As taught by Bland, there would be no expectation of success for using organic acids without sufficient amounts of formaldehyde. The currently claimed invention does not recite or require any formaldehyde, and formaldehyde fails to qualify as a compound of formula (I).

Paquet Has Not Been Shown to Disclose or Teach a Compound of Formula (I)

The Office asserts that Paquet discloses a compound that reads on formula (I).[70] Nevertheless, the Office has not demonstrated a prima facie case of obviousness, as no particular N-acyl methionine compound from the Paquet reference has been identified that meets the limitations of formula (I). It has not been shown which, if any, of the X and Y constituents of the formula X-CO-NH-Y disclosed in Paquet would meet the limitations of formula (I). [71]

The Office has also not articulated any reason why formula (I) would have been obvious in view of the formula disclosed by Paquet. Several teachings of Paquet are generally inconsistent with formula (I), including the preferred embodiments wherein the X group is selected from large acyl groups, such as sorbyl or fatty acyl (C8-C24). These particular acyl chains would not give one of skill in the art any motivation to modify the compound to arrive at formula (I). As such, Paquet does not provide the requisite motivation to combine or expectation of success with either the generic formula (I) or the specifically recited members of formula (I) such as 2-hydroxy-4-(methylthio)butanoic acid. Consequently, Appellants respectfully assert that a *prima facie* case of obviousness has not been established.

In addition to the above, **Paquet fails to suggest or teach any combination of organic acids, as required by the Group I claims**. (Emphasis

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[70] See, *e.g.*, Final Action at page 11, lines 6-8.

[71] Paquet requires X to be an acyl moiety and Y to be a D-amino acid or glycine moiety. It has yet been shown by the Office that an N-acyl methionine would, in fact, read on the compound of formula (I)

Added). The Office acknowledges this failure at page 11 of the Final Action, lines 20-21, "**<u>Paquet et al. does not specifically teach the combination of N-acyl methionine with other organic acids</u>**." (Emphasis Added). In fact, the only alternative antimicrobial compounds suggested by Paquet are mutagenic or toxic, and which are considered unsuitable for food or water. One of skill in the art would not be motivated to combine the disclosure of Paquet with Bland. As stated previously, Bland teaches that the individual organic acids recited in claims 75-82 and 96 are generally ineffective as bacteriocidal agents in animal feedstuffs without large amounts of formaldehyde. Because Paquet and Bland do not disclose the claimed methods or provide any motivation for one skilled in the art to modify the prior art, Appellants respectfully submit that claim 75-82 and 96 are patentable over the cited art.

Accordingly, the rejection of claims 75-82 and 96 under 35 U.S.C. 103(a) over Paquet and Bland is improper.

G. The Rejection of Claims 75, 77, 97, 99-103, 113-117, and 133 under 35 U.S.C. 103(a) over Doerr *et al.* ("Doerr") and Rolow is Improper

1. The Group I Claims Under Rejection (Claims 75, 77, 97, 99-103, and 133)

Claim 75 is representative of the Group I claims. Claim 75 is directed to a method of killing microbes in food or water. The method comprises treating the food or water with an antimicrobial composition. The antimicrobial composition comprises at least two organic acids selected from the group consisting of formic acid, butyric acid, fumaric acid, lactic acid, benzoic acid, and propionic acid; and a third organic acid that is a compound of formula (I). The arguments asserted above in Parts B-F are hereby reasserted with respect to Part G(1).

The Office recites that "Doerr *et al.* discloses that 2-hydroxy-4-(methylthio)butanoic acid may reduce mold growth in corn."[72] However, nowhere does Doerr disclose any combination of 2-hydroxy-4-(methylthio)butanoic acid

[72] See, *e.g.*, Final Action at page 13, line 1.

with an organic acid, let alone the at least two recited in the Group I claims. The Office acknowledges this failure: "**Doerr *et al.* does not specifically teach *the combination* of hydroxyl-methylthio butanoic acid with other organic acids**, and acidulant such as phosphoric acid."[73] In addition, the Office further admits that "**Doerr *et al.* do not teach *the particular amounts* of 2-hydroxy-4-(methylthio)butanoic acid, organic acids and acidulant**."[74] As such, Doerr *et al.* fails to suggest a compound with formula (I) with at least two organic acids selected from the group consisting of formic acid, butyric acid, fumaric acid, lactic acid, benzoic acid, and propionic acid.

It has been previously shown that Rolow is limited to tortillas and products made from tortilla flour.[75, 76] Rolow indicates that a number of known antimicrobial preservatives, including those claimed by Appellants, are unacceptable for individual use in tortillas because they adversely affect taste and odor.[77] **Rolow specifically identifies fumaric acid and benzoic acid to be unsatisfactory as individual antimicrobial agents in tortillas, giving an off flavor and being ineffective at controlling growth of high level organisms.**[78, 79] (Emphasis Added). Fumaric acid and benzoic acid are two of

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[73] See, *e.g.*, *id.* at page 13, lines 4-6. (Emphasis Added).
[74] See, *e.g.*, *id.* at page 13, lines 7-8. (Emphasis Added).
[75] "[I]t can be seen that the combination of benzoic acid with propionic acid and phosphoric acid, in the proportions specified, is an effective preservative for products made from tortilla flour." (See, e.g., Rolow *et al.* at col.7, lines 47-51).
[76] "This invention relates generally to methods and chemicals for extending the shelf life of corn tortillas or wheat tortillas, and specifically the preservation of corn tortillas or wheat tortillas . . ." (See, *e.g.*, *id.* at col. 1, lines 12-15).
[77] "Various antimicrobial preservatives have been proposed, however they have limitations of increasing the cost of producing tortilla and/or adversely affecting the taste and odor." (See, *e.g.*, *id.* at col. 1, lines 35-38).
[78] "[A]cidulants such as fumaric acid or citric acid, are used to reduce pH levels. A major drawback resulting from this type of preservative mixture is the lingering after-taste of the acidulant. These preservative mixtures have successfully increased the shelf life of tortillas . . . However, the taste of the tortillas containing these preservatives has not been satisfactory. Also the supply of some of these preservatives have been limited, making them difficult to or expensive to obtain." (See, *e.g.*, *id.* at col. 2, lines 5-13).
[79] "Benzoic acid is a well-known food preservative . . . generally used only in very acidic foods such as pickles, soft drinks and dressings. . . Benzoic acid is also known to impart an off flavor. Because of the narrow pH range in which it has generally been effective and because of its off-flavor, it is being replaced by other preservatives. Benzoic acid has not been effective to control the growth of high-levels of microorganisms. Because tortillas generally have a pH level above

the organic acids specifically recited in the Group III claims. The teachings away by Rolow may not be disregarded, since two of the "primary indications of spoilage in tortillas is an off odor or taste . . ."[80] One of skill in the art would therefore view Rolow as describing an **unusual combination that acts contrary to the prior art and contrary to the individual properties of the ingredients**.[81]

Ivey, Blake, and Bland have all shown there is significant unpredictability in the microbial arts, and each reference teaches away from any motivation to combine or expectation of success regarding the currently claimed invention. Ivey has shown that a combination of Alimet and propionic acid may be used a vehicle for delivering microbes, contradicting the Office's contention that each component of Appellants' claimed invention is *necessarily* antimicrobial. Regarding the rejection of claims 75, 77, 97, 99-103, and 133, it is entirely unclear whether the addition of another chemical, such as a compound of formula (I), would disturb the unusual properties of the Rolow antimicrobial composition. Rolow provides no teachings whatsoever regarding a compound of formula (I), or whether it would be compatible with any given acid combination.

The Office has not set-forth any sufficient art-based rationale as to why a person of skill in the art would have been motivated to combine 2-hydroxy-4-(methylthio)butanoic acid as disclosed by Doerr, along with the recited tortillas shell preservatives disclosed by Rolow. As shown above, the Bland patent expressly teaches that organic acids and their combinations are generally ineffective as bacteriocidal agents in animal feedstuffs without large amounts of formaldehyde.[82] This teaching away by Bland undercuts any supposed motivation to combine the references cited in this rejection, and supports a finding of non-obviousness. As shown previously and reasserted here, Ivey shows that Alimet and propionic acid are not sufficient to inhibit or kill microbes in food, but instead may be used for the contrary purpose of a delivery vehicle for

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the optimum effective antimicrobial range of benzoic acid, benzoic acid has not been commonly used as a tortilla preservative." (See, *e.g.*, *id.* at col. 3, lines 12-27) (internal citations omitted).
[80] See, *e.g.*, *id.* at col. 1, lines 35-38.
[81] *Id.*
[82] See, e.g., Bland et al. at column 2, lines 18- 26. (Emphasis Added).

microbes. The Ivey reference also indicates that there may be an infinite number of potential food additives including, *e.g.*, proteins, acids, antibiotics, vitamins, and minerals, and for which there is no indication which combination may be successful or detrimental at inhibiting microbes in food or water. Finally, the Office has not established that a skilled artisan at the time of filing would have had a reasonable expectation of success even if its proposed combination were made.

Accordingly, the rejection of claims 75, 77, 97, 99-103, and 133 under 35 U.S.C. 103(a) over Doerr and Rolow is improper.

2. The Group III Claims Under Rejection (Claims 113-117)

Claim 113 is representative of the Group III claims under rejection. Claim 113 is directed to a method of killing microbes in food or water. The method comprises treating the food or water with an antimicrobial composition. The antimicrobial composition comprises at least two organic acids selected from the group consisting of formic acid, butyric acid, fumaric acid, lactic acid, benzoic acid, and propionic acid; and a third organic acid that is a compound of formula (I), **wherein the compound of formula (I) is 2-hydroxy-4-(methylthio)butanoic acid**. (Emphasis Added).The arguments asserted above in Parts B-G(1) are hereby reasserted with respect to Part G(2) and claims 113-117.

The Rolow *et al.* reference is cited by the Office as allegedly disclosing a liquid preservation composition to extend the shelf life of tortillas made from corn. In a preferred embodiment, the preservation composition of Rolow is said to comprise "phosphoric acid, propionic acid, and benzoic acid."[83] Rolow does not teach, disclose, or suggest any compound of formula (I) as claimed by the Appellants.

Doerr fails to suggest or teach any combination of organic acids for use in the preservation of food and water. Neither reference provides a motivation to

[83] See, *e.g.*, Final Action at page 9, lines 18-21.

combine. It has been previously been shown by Bland that the organic acids disclosed by Rolow are not effective at killing microbes in animal feedstuffs. This teaching away by Bland contradicts any supposed motivation to combine of Rolow and Doerr or any expectation of success, and supports a finding of non-obviousness.

Accordingly, the rejection of claims 113-117 under 35 U.S.C. 103(a) over Doerr *et al.* and Rolow *et al.* is improper.

H. Conclusion

For the foregoing reasons, the appellants respectfully submit that claims 75, 77-104, and 113-133, as amended, satisfy 35 U.S.C. § 112, second paragraph and request that the rejection of these claims as having uncertain scope be reversed. The appellants also respectfully submit that claims 75, 77-104, and 113-133 are patentable over the prior art, and request that the rejection of these claims as being unpatentable under 35 U.S.C. § 103 (a) be reversed. The Commissioner is hereby authorized to change any and all fees that may be required or credit any overpayment to Deposit Account No. 50-1662.

Polsinelli Shalton Flanigan Suelthaus PC

Respectfully submitted,

Date: August 20, 2008

By: /Kathryn J. Doty/

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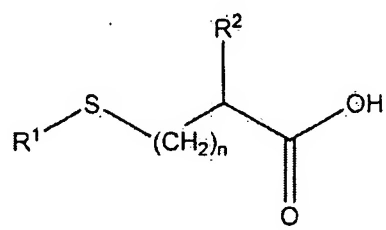
Attorney for Appellants

Claims Appendix to Appeal Brief Under Rule 47.37(c)(1)(viii)

Claims1-74 (withdrawn).

Claim 75 (previously presented). A method of inhibiting or killing microbes in food or water, the method comprising treating the food or water with a composition, the composition comprising at least two organic acids selected from the group consisting of formic acid, butyric acid, fumaric acid, lactic acid, benzoic acid, and propionic acid; and a compound of formula (I) having the following structure:



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wherein R^1 is an alkyl group having from one to four carbon atoms;

n is an integer from 0 to 2;

R^2 is selected from the group consisting of hydroxy, amino, --$OCOR^3$, or --$NHCOR^3$; and

R^3 is an organic acid derivative;

or a salt thereof;

and an acceptable diluent, adjuvant or excipient.

Claim 76 (canceled).

Claim 77 (previously presented). The method of claim 75 wherein said food is selected from the group consisting of human food, livestock food, pet food, or aquaculture food.

Claim 78 (previously presented). The method of claim 77 wherein said composition is mixed with the food as it is formulated.

Claim 79 (previously presented). The method of claim 78 wherein said composition is applied to a pre-mixed or pre-pelleted feed.

Claim 80 (previously presented). The method of claim 79 wherein said composition, subsequent to treating said food, is uniformly dispersed throughout said food.

Claim 81 (previously presented). The method of claim 75 wherein said food comprises a meat or bone meal.

Claim 82 (previously presented). The method of claim 75 wherein said food is dry food.

Claim 83 (previously presented). The method of claim 75 wherein said food is liquid food.

Claim 84 (previously presented). The method of claim 75 wherein said food is a combination of dry feed and liquid food.

Claim 85 (previously presented). The method of claim 75 wherein said food is fed to an animal.

Claim 86 (previously presented). The method of claim 85 wherein said animal is a ruminant animal.

Claim 87 (previously presented). The method of claim 86 wherein said ruminant animal is selected from the group consisting of dairy cows, lactating dairy cows, dairy calves, beef cattle, sheep, and goats.

Claim 88 (previously presented). The method of claim 85 wherein said animal is an aquaculture.

Claim 89 (previously presented). The method of claim 88 wherein said aquaculture is fish or crustaceans.

Claim 90 (previously presented). The method of claim 85 wherein said animal is livestock.

Claim 91 (previously presented). The method of claim 90 wherein said livestock is swine or horses.

Claim 92 (previously presented). The method of claim 85 wherein said animal is poultry.

Claim 93 (previously presented). The method of claim 92 wherein said poultry is selected from the group consisting of chickens, turkeys, and hatchlings thereof.

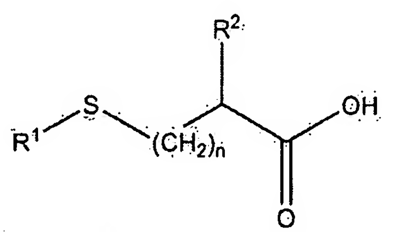
Claim 94 (previously presented). The method of claim 85 wherein said animal is a companion animal.

Claim 95 (previously presented). The method of claim 94 wherein said companion animal is a dog or a cat.

Claim 96 (previously presented). The method of claim 75 wherein said microbe is a bacterium.

Claim 97 (previously presented). The method of claim 75 wherein said microbe is a mold.

Claim 98 (previously presented). A method of killing mold in food or water comprising corn and soy, the method comprising apply to said food or water a composition, the composition comprising at least two organic acids selected from the group consisting of formic acid, butyric acid, fumaric acid, lactic acid, benzoic acid, and propionic acid; and a compound of formula (I) having the following structure:



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wherein R^1 is an alkyl group having from one to four carbon atoms;

n is an integer from 0 to 2;

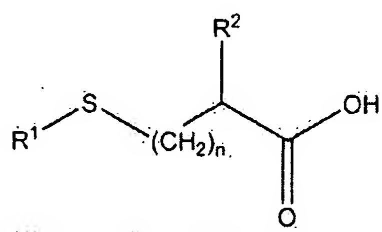
R^2 is selected from the group consisting of hydroxy, amino, --OCOR^3, or --NHCOR^3; and

R^3 is an organic acid derivative;

or a salt thereof;

and an acceptable diluent, adjuvant or excipient.

Claim 99 (previously presented). A method of killing mold in food having a moisture content of from 0-17%, the method comprising applying to said food a composition, the composition comprising at least two organic acids selected from the group consisting of formic acid, butyric acid, fumaric acid, lactic acid, benzoic acid, and propionic acid; and a compound of formula (I) having the following structure:



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wherein R^1 is an alkyl group having from one to four carbon atoms;

n is an integer from 0 to 2;

R^2 is selected from the group consisting of hydroxy, amino, --OCOR^3, or --NHCOR^3; and

R^3 is an organic acid derivative;

or a salt thereof;

and an acceptable diluent, adjuvant or excipient.

Claim 100 (previously presented). The method of claim 99 wherein said moisture content is at least 0.01% by weight of the food.

Claim 101 (previously presented). The method of claim 99 wherein said moisture content is at least 1% by weight of the food.

Claim 102 (previously presented). The method of claim 99 wherein said moisture content is at least 5% by weight of the food.

Claim 103 (previously presented). The method of claim 99 wherein said moisture content is at least 10% by weight of the food.

Claim 104 (previously presented). The method of claim 75 wherein said animal food is heat-treated, either before or after application of said composition.

Claim 105-112 (canceled).

Claim 113 (previously presented). The method of claim 75, wherein the compound of Formula I is 2-hydroxy-4-(methylthio)butanoic acid.

Claim 114 (previously presented). The method of claim 113, further comprising an acidulant selected from the group consisting of phosphoric acid, sulfuric acid, phosphorous acid, hydrochloric acid, hydrobromic acid, and nitric acid.

Claim 115 (previously presented). The method of claim 113, wherein the composition has a pH of less than about 5.

Claim 116 (previously presented). The method of claim 113, wherein the composition has a pH of about 4 to about 5.

Claim 117 (previously presented). The method of claim 113, wherein the composition has a pH of about 4.5.

Claim 118 (previously presented). The method of claim 75, wherein the compound of Formula I is 2-hydroxy-4-(methylthio)butanoic acid; and the organic acids consist of formic acid and propionic acid

Claim 119 (previously presented). The method of claim 118, wherein the content of 2-hydroxy-4-(methylthio)butanoic acid is from about 5% to about 20% of the sum of the 2-hydroxy-4-(methylthio)butanoic acid, formic acid, and propionic acid content; the content of the formic acid is from about 65% to about 85% of said sum; and the content of the propionic acid is from about 1% to about 15% of said sum.

Claim 120 (previously presented). The method of claim 119, further comprising phosphoric acid, wherein the content of the phosphoric acid is from about 5% to about 20% of said sum.

Claim 121 (previously presented). The method of claim 75, wherein the compound of Formula I is 2-hydroxy-4-(methylthio)butanoic acid; and the organic acids consist of butyric acid, and lactic acid.

Claim 122 (previously presented). The method of claim 121, wherein the content of 2-hydroxy-4-(methylthio)butanoic acid is from about 20% to about 40% of the sum of the 2-hydroxy-4-(methylthio)butanoic acid, butyric acid, and lactic acid content; the content of the butyric acid is from about 10% to about 30% of said sum; and the content of the lactic acid is from about 10% to about 30% of said sum.

Claim 123 (previously presented). The method of claim 122, further comprising phosphoric acid, wherein the content of the phosphoric acid is from about 20% to about 40% of said sum.

Claim 124 (previously presented). The method of claim 75, wherein the compound of Formula I is 2-hydroxy-4-(methylthio)butanoic acid; and the organic acids consist of butyric acid, formic acid, and lactic acid.

Claim 125 (previously presented). The method of claim 124, wherein the content of 2-hydroxy-4-(methylthio)butanoic acid is from about 10% to about 30% of the sum of the 2-hydroxy-4-(methylthio)butanoic acid, butyric acid, formic acid, and lactic acid content; the content of the butyric acid is from about 2% to about 22% of said sum; the content of the formic acid is from about 20% to about 40% of

said sum; and the content of the lactic acid is from about 8% to about 28% of said sum.

Claim 126 (previously presented). The method of claim 125, further comprising phosphoric acid, wherein the content of the phosphoric acid is from about 10% to about 30% of said sum.

Claim 127 (previously presented). The method of claim 75, wherein the compound of Formula I is 2-hydroxy-4-(methylthio)butanoic acid; and the organic acids consist of butyric acid, lactic acid, and propionic acid.

Claim 128 (previously presented). The method of claim 127, wherein the content of 2-hydroxy-4-(methylthio)butanoic acid is from about 10% to about 30% of the sum of the 2-hydroxy-4-(methylthio)butanoic acid, butyric acid, lactic acid, and propionic acid content; the content of the butyric acid is from about 2% to about 22% of said sum; the content of the lactic acid is from about 8% to about 28% of said sum; and the content of the propionic acid is from about 20% to about 40% of said sum.

Claim 129 (previously presented). The method of claim 128, further comprising phosphoric acid, wherein the content of the phosphoric acid is from about 10% to about 30% of said sum.

Claim 130 (previously presented). The method of claim 75, wherein the compound of Formula I is 2-hydroxy-4-(methylthio)butanoic acid; and the organic acids consist of butyric acid, formic acid, and propionic acid.

Claim 131 (previously presented). The method of claim 130, wherein the content of 2-hydroxy-4-(methylthio)butanoic acid is from about 1% to about 20% of the sum of the 2-hydroxy-4-(methylthio)butanoic acid, butyric acid, formic acid, and propionic acid content; the content of the butyric acid is from about 1% to about 15% of said sum; the content of the formic acid is from about 65% to about 85% of said sum; and the content of the propionic acid is from about 1% to about 15% of said sum.

Claim 132 (previously presented). The method of claim 131, further comprising phosphoric acid, wherein the content of the phosphoric acid is from about 1% to about 15% of said sum.

Claim 133 (previously presented). The method of claim 113, wherein the composition has an improved odor.

<u>Evidence Appendix to Appeal Brief Under Rule 47.37(c)(1)(ix)</u>

A copy of Dr. Knight's Declaration under 37 C.F.R. 1.132 was submitted to the USPTO on September 26, 2007, as part of the response to the non-final Office Action mailed March 27, 2007. The response was entered by the Examiner as indicated by the Final Action mailed December 11, 2007. The Final Action made specific reference to the entry of Dr. Knight's Declaration at page 16, lines 14-23, page 17, lines 1-7. A copy of Dr. Knight's Declaration is hereby attached as evidence to the Appeal Brief.

A copy of the Warnecke *et al.* review article under 37 C.F.R. 1.132 was submitted to the USPTO on September 26, 2007, as part of the response to the non-final Office Action mailed March 27, 2007. The response was entered by the Examiner as indicated by the Final Action mailed December 11, 2007. The Final Action did not make specific reference to the Warnecke *et al.* review article, but generally discussed entry of the Appellants submitted evidence at page 16, lines 14-23, page 17, lines 1-7. A copy the Warnecke *et al.* review article is hereby attached as evidence to the Appeal Brief.

Related Proceedings Appendix to Appeal Brief Under Rule 47.37(c)(1)(x)

There are no related decisions for this appeal.

UNITED STATES PATENT AND TRADEMARK OFFICE

Applicant(s):	Schasteen et al.	Art Unit	1617
Serial No.:	10/652,745	Examiner:	S. Kantamneni
Filed:	August 29,2003	Conf. No.	1765
For:	ANTIMICROBIAL COMPOSITIONS		

DECLARATION OF CHRISTOPHER D. KNIGHT UNDER 37 C.F.R. § 1.132

I, Christopher D. Knight, declare and state as follows:

1. I have over twenty years of experience in the field of animal health and nutrition. Novus International Inc., a global leader in animal health and nutritional products, currently employs me as Vice-President for Research and Development. My employment by Novus International has been continuous for over sixteen years. Prior to my employment at Novus International Inc., I was employed by Monsanto in their Animal Sciences Division for over five years. My educational background includes a Bachelor of Science degree in Animal science awarded by Cornell University in 1975; a Master of Science degree in Monogastric Nutrition awarded by Purdue University in 1977; and a doctorate degree (i.e., Ph.D.) in Monogastric Nutrition awarded by Purdue University in 1981. I have also published over approximately thirty journal articles or posters at internationally attended meetings, and I am an inventor on three patents. Attached to this Declaration is a copy of my curricula vitae.

2. I have reviewed U.S. Patent Application Publication No. 2004/0175434 ('434 application) entitled "Antimicrobial Compositions." The '434 application has claims directed toward antimicrobial compositions that comprise several organic acid formulations developed at Novus, and presently sold under the trade name ACTIVATE®.

3. Through my position at Novus as Vice-President for Research and Development, I am familiar with and supervised portions of the research and development efforts that resulted in the discovery of several organic acid blends, which are claimed in the '434 application. The focus of this research effort was to improve the cost effectiveness of the formulations, while at the same time improving the antimicrobial activity of the blend of organic acids compared to any individual organic acid comprising the blend. The ACTIVATE® organic acid

formulations (as described in various iterations of the '434 application), in my opinion, meet both of the aforementioned goals.

4. We have research data, that in my opinion, demonstrates surprising and unexpected results for organic acid formulations falling within the scope of the '434 patent claims. As an example, attached to this Declaration is a graph (identified as figure 7) that depicts a synergistic effect for two organic acid formulations of the claimed invention. With reference to the attached graph, data is depicted for the antimicrobial activity of five different organic acid compositions against *Salmonella* in feed. The five organic acid compositions include: (1) 0.45% HMTBA alone (i.e., 2-hydroxy-4-(methylthio)butanoic acid, which is a compound of Formula (I) in the '434 application); (2) 0.45% butyric acid alone; (3) 0.45% lactic acid alone; (4) blend OA 4, which is 0.15% lactic acid, 0.15% propionic acid, and 0.15% HMTBA; and (5) blend OA 6, which is 0.1% lactic acid, 0.1% butyric acid, 0.1% propionic acid, and 0.15% HMTBA. The antimicrobial experiments were conducted in accordance with Novus's standard protocol entitled "Low pH in Feed Test Procedure," a copy of which is attached to this Declaration. As depicted in the graph, the antimicrobial activity of either blend OA 4 or blend OA 6 achieved significantly higher killing of *Salmonella* at lower concentrations than could be achieved with any of the single organic acids alone.

5. I further declare that all statements made herein are of my own knowledge are true and that all statements made on information and belief are believed to be true; and further, that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under 18 U.S.C. § 1001, and that such willful false statements may jeopardize the validity of the application or any patent issuing thereon.

Christopher D. Knight
Christopher D. Knight

9/25/2007
Date

CURRICULUM VITAE

Christopher D. Knight, Ph.D
31 Ranch Court
St. Louis, MO 63146
(314) 567-6627 (h)
(636) 926-7401 (o)

Education

1977- 1981	Ph.D. in Monogastric Nutrition Purdue University, West. Lafayette, IN Department of Animal Science. Graduate Instructorhsip, 1977-1981
1975- 1977	M.S. in Monogastric Nutrition Purdue University, West. Lafayette, IN Department of Animal Science. Graduate Research Assistant.
1973- 1975	B.S. Animal Sciences Cornell University, Ithaca, NY
1971- 1973	A.A.S. Science Laboratory Technology State University of New York at Cobleskill

Employment

2001- Present	Department Head, Research & Development Novus International, Inc.
1996- 2001	Director New Business Development Novus International, Inc.
1991- 1995	Manager and Director Nutrition Research Novus International, Inc
1987- 1991	Research Group Leader Monsanto Company Animal Sciences Division Porcine Somatotropin Group
1981- 1986	Research Specialist and Research Group Leader Monsanto Company Alimet Metabolism and Applications Research Group

Key Accomplishments

- Developed foundation data quantifying availability of ALIMET® Feed Supplement as a by-pass methionine source in lactating dairy cattle and methods to predict methionine deficiency using existing nutritional models. These data resolved decades of research work to attempting to commercialize this product application that had failed due to unpredictable field results. The research demonstrated Alimet to be the most cost-effective source of post-ruminal methionine activity available, resulted in a US patent and the development of a $5M/yr business for Novus. As of 2005, a new Ruminant Business Unit of 20 employees and agents and a portfolio of 8 products (including Alimet and MHA) for the dairy industry has been formed.

- Led the development and commercialization of OASIS® Hatchling Supplement, a hydrated nutritional supplement fed to young poultry in transit or to stimulate rapid onset of ad libitum feeding after placement. This patented product developed a new market in the poultry industry based on developmental research at Novus showing the impact of early nutrition on subsequent long term performance and health. Cumulative sales of this niche product have exceeded $4M and resulted in the development of gastrointestinal health as a core research and development competency within Novus.

- Led the technology development, regulatory approval and early commercialization of ADVENT® Coccidiosis Control, an orally applied coccidiosis vaccine based upon technology that permits the in vitro determination of oocyst viability such that a vaccine of consistent potency can be produced and marketed. This represented a new area of technology for Novus and in 2003, a jury of scientists and technology experts from Washington University and St. Louis University awarded the developers of this technology (Dr. Julia Dibner and Dr. Chris Knight) with The St. Louis Technology Award. The Advent Coccisosis Control technology was among eight other winners from approximately 70 nominations in the St. Louis vicinity. In determining winners, the judges considered the scope, economic impact and overall significance of the new technology. Facilitated by the Academy of Science of St. Louis, the judging process also examined the level of sophistication of the entries and the innovation utilized to bring it to fruition. This technology represents a keystone of a business strategy that focuses on gastrointestinal health and drug-free poultry production.

- Established a new cost-efficient method of product development research, to insure Novus' capability to conduct scientifically and commercially relevant research across multiple species without requiring ownership or hands on care and management of research facilities. Initially divested Novus-owned animal research facilities and sought collaborative investment opportunities with scientific professionals in animal agriculture to provide capital for research facilities that would be controlled by the research partner but provide Novus with preferred status for conduct of research. To date we have formed 3 partnerships like this in the US that permits routine product development work in broilers, swine (weaning, grow-finish and lactating sows) and dairy cattle, all in commercial scale production environments. Similar agreements are

under development in Brazil (commercial scale egg layer research) and China (commercial scale swine research including wean, grow-finish and sow nutrition).

- The foundation product for Novus International is ALIMET® Feed Supplement, a source of methionine activity referred to as methionine hydroxyl analog or chemically DL-2-hydroxy-4-(methylthio) butanoic acid. Today this business represents approximately $400M in annual revenue to Novus in a $1B methionine market; however, in 1981 this represented about a $20M business. In the course of my 25 year involvement with this product there has been a heated commercial controversy with respect the relative efficacy of Alimet and the competitive product DL-methonine (DLM). A close colleague (Dr. Julia Dibner) and I have had the responsibility of understanding the absorption, metabolism and utilization of Alimet, how it differs from that of DLM and the impact that the differences have on the commercial value of Alimet relative to DLM. Today based on a variety of independent and collaborative research efforts it is understood that the metabolism of Alimet is very different from DLM, that those differences result in differences in ad libitum feed intake (less than DLM at low supplementation rates, greater than DLM at the maximum response level) resulting in different dose responses for the two methionine sources. A substantial part of the controversy was based on the a priori assumption that the two products must have the same dose response since they both provide methionine. With collaboration with various statistical experts, we have been able to establish that the two products in fact have different dose responses and have described the appropriate statistical methods for comparing two products that exhibit different dose responses (Poult. Sci. 85:947-954). The controversy will continue due to commercial conditions (Alimet is less expensive to manufacture than DLM) , however over the course of 25 years Alimet has continued to grow at a 25% compounded annual growth rate with over a 50% market share in the US. The science applied to this commercial issue has laid the technical foundation that has provided Novus with the technical credibility to expand our product offerings from amino acids into nutritional organic acid blends, organic trace minerals, ingredient preservation and coccidiosis control.

ALIMET® Feed Supplement, OASIS® Hatchling Supplement and ADVENT® Coccidiosis Control are registered trademarks of Novus International, Inc., St. Louis, MO.

Personal

- Married 1982: Sandra J. Rogers (Purdue Food Science MS 1978).
- Children: Adam (19), Evan (16), Audrey (14)

Community Involvement

- Subdivision Trustee: 1987-1989: Led resolution of road and storm sewer repair dispute
- St. Peter's Episcopal Church:
    - Youth Sponsor: 1984-1988
    - Sunday School Teacher:1992-2006 (Variety of grades and curricula)
    - Vestry: 1989-1993
    - Founding Christian Education Commission & Chair: 1989-1993
    - Confirmation Teacher: 2005-6.
    - Founding and sustaining member of Haven of Grace: Home for unwed mothers
- Hobbies
    - Cooking
    - Gardening
    - Kid's Sports

PUBLICATIONS & PROCEEDINGS

1. Dibner, J.J. and **C.D. Knight** (1984) Conversion of 2-hydroxy-4-(methylthio) butanoic acid to L-methionine in the chick: A stereospecific pathway. J. Nutr. 114:1716-1723.

2. **Knight, C.D.** and J.J. Dibner (1984) Comparative absorption of 2-hydroxy-4-(methylthio)butanoic acid and L-methionine in the broiler chick. J. Nutr. 114:2179-2186.

3. Dibner, J.J., F.J. Ivey, C.Q. Lawson and **C.D. Knight** (1986) In *vitro* methods in animal nutrition. Proceedings of the Conference European D'Aviculture 7:312-316.

4. Dibner, J.J., **C.D. Knight**, R.A. Swick and F.J. Ivey (1987) Absorption of 2-hydroxy-4-(methylthio) butanoic acid from the hindgut of the broiler chick. Poult. Sci. 67:1314-1321.

5. Dibner, J.J., **C.D. Knight**, C.Q. Lawson, R.A. Swick and F.J. Ivey (1990) Studies of the metabolism of 2-hydroxy-4-(methylthio)butanoic acid (HMB, Alimet®) in the broiler chick using *in vitro* methods. Memorias: XI Congreso de Avicultura Centroamericano y del Caribe, pp15-18.

6. **Knight, C.D.**, J.J. Dibner and F.J. Ivey (1991) Crystalline amino acid diets for chicks: History and future. Maryland Nutrition Conference Proceedings pp 19-28.

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Patents

1. Ivey, F.J., J.J. Dibner, and **C.D. Knight**, 1999. Nutrient formulation and process for enhancing the health, livability, cumulative weight gain or feed efficiency in poultry and other animals. Patent number 5,976,580.

2. Ivey, F.J., J.J. Dibner, and **C.D. Knight**, 1999. Nutrient formulation and process for feeding young poultry and other animals. Patent number 5,985,336.

3. **Knight, C.D.**, K. Koenig, L. Rode, M. Vandenberg, and M. Vázquez-Añón 2000. Process for optimizing milk production. Patent number 601,753

TITLE: Low pH in Feed Test Procedure

METHOD NO.

MATERIAL: Activate DA™

TEST: Anti-bacterial activity of organic acids measured in feed at low pH

SCOPE: Anti-bacterial activity of organic acids is measured in feed at low pH to simulate the low pH and moisture conditions in the upper digestive tract of animal.

MATERIALS:

1. Finished feed: mash or crumble, swine or poultry
2. Fresh culture of *Salmonella* and *Escherichia coli*
4. Brilliant Green Agar or other selective media for salmonella enumeration
5. MacConkey Agar or other selective media for e. coli enumeration
6. Incubator set at 40C for the assay, and 37C for bacteria enumeration (plating)
7. Pipettes and sterile tips
8. Sterile tubes (50 ml)
9. Hydrochloric acid

SAFETY CONSIDERATIONS:

1. Mouth pipetting is not allowed, automatic pipettes or pipette bulbs must be used.
2. Use appropriate gloves where necessary.
3. Dispose of all hazardous waste properly. Autoclave all wastes containing salmonella or e. coli.

PROCEDURE:

Prepare fresh cultures of salmonella and e. coli:

1. Grow a fresh culture of salmonella or e. coli overnight at 37C in Tryptic Soy Broth (or appropriate media for the particular strain of bacteria)
2. Determine the counts by direct plating
3. Keep the culture at 4C until use. Prepare fresh cultures every 2 weeks.

Determine the amount of HCL needed to bring the feed to pH 4.0

1. Prepare 150mM HCL solution from concentrated HCl (12.1N HCl),
2. Weight out 5g of mash or crumbled feed in 50ml tubes,
3. Add 150mM HCl and DI H2O at different proportions (see the table below) to achieve a total volume of 15 ml,

150mM HCl	7.25 ml	7.50ml	7.75 ml	8 ml	8.25ml
DI H2O	7.75 ml	7.50ml	7.25 ml	7 ml	6.75ml
Total volume	15 ml	15ml	15 ml	15 ml	15 ml

4. Vortex the samples for ~1 min, keep at 40C for ~20min (preferable with mixing) for the pH to equilibrate,

5. Adjust the ratio between HCl and H2O until the pH of the feed is at ~ 4.0 (A range of 3.8 to 4.0 is acceptable).

Set up the following treatments (in 50 ml sterile tubes):

	Treatments	Dose	Reps.	Feed	Inoculant (cfu/g of feed)
1	control		2-3	5 gram	40,000
2	Activate DA	0.3%	2-3	5 gram	40,000
3	Activate DA	0.5%	2-3	5 gram	40,000

1. Weigh out 5g of finished feed in a sterile 50 ml centrifuge tube.
2. Add Activate DA to treatments 2 and 3 (15mg in the 0.3% treatment, and 25mg in the 0.5% treatment).
3. Add HCl and DI H2O to bring the pH to 4.0 (pre-determined for each feed, see the procedures above),
4. Inoculate with Salmonella or E. coli to give a final concentration of 40,000 cfu per ml of sample (40,000 cfu/ml x 15 ml = 600,000 cfu/tube).
5. Incubate the samples for 90 minutes in a 40C incubator (preferably with mixing on an end to end rotator, but not required).
6. At the end of 90 minutes incubation, prepare 1:10 dilution of sample in sterile H2O (1ml sample and 9 ml H2O)
7. Plate the following samples on Brilliant Green agar (*salmonella*) and MacConkey agar (*E. coli*) and incubate plates at 37C overnight.
   100ul of 1:10 dilution from step 6
   100ul of undiluted sample
8. Count colonies the next day, determine cfu/ml sample, and compare with control.

ANALYTICAL TIME:

REFERENCE:
ATTACHMENTS : None

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Organic acid toxicity, tolerance, and production in *Escherichia coli* biorefining applications

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Abstract

Organic acids are valuable platform chemicals for future biorefining applications. Such applications involve the conversion of low-cost renewable resources to platform sugars, which are then converted to platform chemicals by fermentation and further derivatized to large-volume chemicals through conventional catalytic routes. Organic acids are toxic to many of the microorganisms, such as *Escherichia coli*, proposed to serve as biorefining platform hosts at concentrations well below what is required for economical production. The toxicity is two-fold including not only pH based growth inhibition but also anion-specific effects on metabolism that also affect growth. *E. coli* maintain viability at very low pH through several different tolerance mechanisms including but not limited to the use of decarboxylation reactions that consume protons, ion transporters that remove protons, increased expression of known stress genes, and changing membrane composition. The focus of this mini-review is on organic acid toxicity and associated tolerance mechanisms as well as several examples of successful organic acid production processes for *E. coli*.

Review

Biorefining Platforms

Biorefining promises the development of efficient processes for the conversion of renewable sources of carbon and energy into large volume commodity chemicals. It has been estimated that such bioprocesses already account for 5% of the 1.2 trillion dollar US chemical market [1], with some projecting future values of up to 50% of the total US chemical market generated through biological means. While the attractiveness of such bioprocesses has been recognized for some time [2,3], recent advances in biological engineering and associated sciences [4-15], several biorefining success stories [16-18], and instability in the price and future availability of oil [19], have collectively reinvigorated interest in the large scale production of chemicals through biological routes. Nevertheless, many challenges still remain for the economical bio-production of commodity chemicals. Such challenges encompass the need to not only inexpensively convert biomass into usable sources of carbon and energy but also to engineer microbes to produce relevant chemicals at high titers and productivities while minimizing the generation of byproducts that might foul downstream processes [1,20,21]. One model for addressing the latter of such challenges involves the generation of platform organisms that can be easily engineered and re-engineered to produce a variety of building block chemicals that are amenable to conversions to higher value products via traditional catalytic routes (see Figure 1). Although chemical pretreatment of raw materials impairs viability of platform organisms, this review will focus on product toxicity issues associated with the production of organic acids in

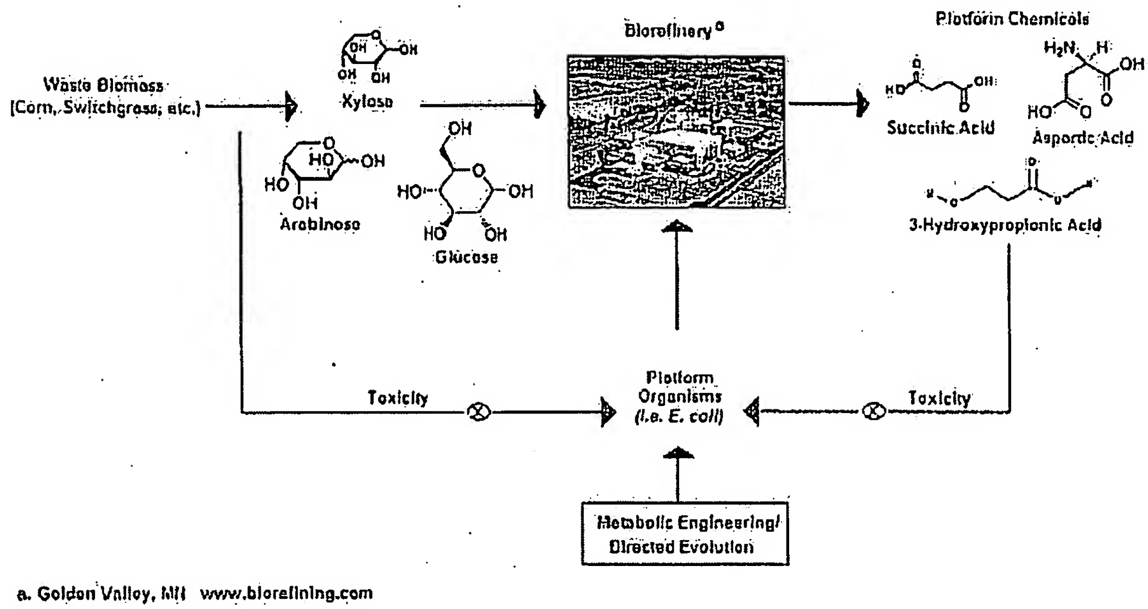

Figure 1
Conceptual model of toxicity in biorefining applications. Sugars are extracted from waste biomass for use as feedstock for platform organisms in a biorefinery. Metabolically engineered microorganisms convert sugars into valuable platform chemicals that are then further derivatized to large-volume chemicals. Product and feedstock toxicity are observed, thus limiting productivity of biorefining applications.

E. coli (for further information on sugar extraction from raw materials see Zaldavar, *et al.* [22] and Knauf, *et al.* [23]).

The US Department of Energy (USDOE) recently released a prioritized list of building block chemicals for future biorefining endeavors. Priority was assigned based on the projected value of the platform chemical and potential derivatives as well as what technological developments were required for the production of the chemical and associated derivatives [21]. The report emphasized the importance of organic acids to the future of biorefining efforts (eight of the top twelve chemicals were organic acids, see Table 1 in additional file 1). The USDOE is not the first to recognize the importance of organic acids. In fact, there is a rich literature describing microbial production of organic acids [17,20,24,25], including several successful commercial bioprocesses [26-28]. Product toxicity is one of the primary challenges in the development of organic acid bioprocesses based on the use of platform host organisms, such as *E. coli*. In particular, while *E. coli* is known to survive very high concentrations of acids (pH = 2) when passing through the mammalian stomach, *E. coli* are surprisingly acid sensitive in exponential phase when cultured planktonically [29,30]. Moreover, undissociated organic acids, which pass freely through the outer and plasma membranes of *E. coli* [31,32], dissociate upon entry into the slightly alkaline cytoplasm releasing protons that lower internal pH (pH_i) and anions that specifically inhibit different aspects of metabolism resulting in impaired growth [33-35]. Titers and productivities of 50–100 g/L and 2–3 g/L·hr are expected for the economical manufacturing of most building block acids by fermentation. The pKa values range from 3–5 for these organic acids, which would result in a pH reduction to around 2.0 for titers of 50 g/L. This highlights a key challenge in the metabolic engineering of organic acid production hosts. That is, high titers result in the addition of protons to the culture, which either result in a decreased pH or the addition of large volumes of base titrant. At low pH, organic acids are undissociated, thus they pass freely through the membrane and inhibit growth. At high pH, the process is less efficient due to base requirements and because export of the organic acid cannot proceed by free diffusion alone (for a more detailed discussion of organic acid export issues see Van Maris *et al.* [36]). What is desired, therefore, is a platform organism that not only produces high levels of organic acid chemicals but also is tolerant to any associated toxicity.

Many microbes are capable of producing platform chemicals by aerobic and anaerobic fermentation processes [22]. L-lactic acid has traditionally been produced by lactic acid bacteria. Although many lactic acid bacteria strains have been studied extensively [37], the ability to produce optically pure L-lactic acid is hampered by the presence of both L and D lactate dehydrogenase genes [38]. Pure L-lactic acid must therefore be produced via another pathway, as the racemic acid product is not useful for downstream conversion into polylactic acid. A number of other microorganisms have been used for industrial fermentation of several of the building block organic acids identified in Table 1. Large scale production of amino acids has been accomplished in *Corynebacteriumglutamicum* [39], succinic acid has been produced by *Actinobacillus succinogenes* [40], and itaconic acid production has been carried out with *Aspergillus terrus* [41]. While successful, the future application of these organisms as platform hosts is limited when compared with *E. coli*. *E. coli* is advantageous as a platform host because it is the most well characterized model organism, it has been used in recombinant processes for over 20 years; there are a wide variety of good genetic tools, and it is sensitive to many antibiotics used in genetic engineering efforts [42]. Moreover, the completion of the *E. coli* genome sequence has already enabled many functional genomics studies and proven useful in metabolic engineering efforts [43]. Finally, *E. coli* grows quickly in minimal media and maintains the ability to metabolize both 5 and 6 carbon sugars, which is a specific advantage over the use of industrially relevant yeast strains [22]. This mini-review will describe the basic mechanisms underlying organic acid toxicity and associated tolerance pathways in *E. coli* followed by a short discussion of several metabolic engineering strategies employed for the production of organic acids in *E. coli*.

Organic Acid Toxicity in E. coli

One of the primary factors contributing to the toxicity of organic acids is their ability to diffuse across *E. coli* cellular membranes when undissociated as opposed to the restricted passage of dissociated protons and anions (see Figure 2) [31,32]. Diffusion of dissociated acids is limited to secondary transport, which is known to involve H+/monocarboxylic acid symporters. However, the detailed mechanism and specificities of the transporters remain unknown [31]. *E. coli* maintain a cytoplasmic pH (pH_i = 7.5) that is most often higher than that of the external media and typically well above the pKa of organic acids [44,45]. As a result, organic acids exist in the dissociated form within the cytoplasm. Thus, diffusing organic acids entering into the cytoplasm will dissociate and disrupt the pHi and anion pool of the cytoplasm. The resulting increase in internal acidity can affect the integrity of purine bases [46] and result in denaturing of essential enzymes inside the cell [35], both of which negatively affect cell viability.

Organic acid anions affect cell growth in a variety of manners. Increased anion concentration has been shown to lead to an increased transport of potassium ions into the cell, which increases turgor pressure [47,48]. To maintain a constant turgor pressure and cell volume, glutamate is transported out of the cell [48]. This transport activity concomitantly disrupts the osmolarity of the cytoplasm, which in turn lowers the cell's growth potential and viability. In addition to this general anion effect, there are also effects specific to each organic acid. It has been proposed that enzymes involved in protein synthesis are sensitive to a combination of two unrelated mechanisms, including the acidification of pHi and the formation of an anionic pool [35]. Although this finding implies that the organic inhibition due to the anion pool could be acid specific, the details describing this dual inhibition mechanism remain unclear. Kirkpatrick et al. reported proteins exhibiting increased expression in response to extracellular acetate [33]. Among these are the OppA transporter, RpoS regulon, several amino acid uptake proteins, DNA binding proteins, and extreme-acid preiplasmic chaperones. Interestingly, when formate was introduced in place of acetate the expression of the previously mentioned proteins was repressed, indicating that the response was anion specific. This finding introduces new challenges in addressing organic acid tolerance. Specifically, it highlights the need to engineer both pH and as well as specific anion tolerance into host organisms.

Finally, production of organic acids might include intermediates that are themselves toxic. For example, 3-hydroxypropionic acid (3HP) is closely related to the antimicrobial compound Reuterin. Reuterin describes the hydroxypropionaldehyde (HPA) system including HPA, HPA dimer, and HPA hydrate. Reuterin is inhibitory to several bacteria, including *E. coli*, at concentrations as low as 0.03–0.05 g/L [49-51]. It is thought that the toxicity could be the result of inhibition of DNA synthesis [52]. It has been postulated that the reactivity of the aldehyde group of HPA causes DNA damage similarly to formaldehyde, which is the aldehyde analog of formic acid [49]. Intermediate toxicity can be managed either by optimization of the production pathway in the host or by engineering tolerance to the intermediate itself.

Organic Acid Tolerance in E. coli

E. coli has a remarkable ability to remain viable under a broad range of pH conditions. This ability is essential for its survival in the mammalian digestive system where pH can vary between pH = 2–8. Several different acid tolerance mechanisms have been identified in *E. coli*. While each mechanism is capable of providing some degree of

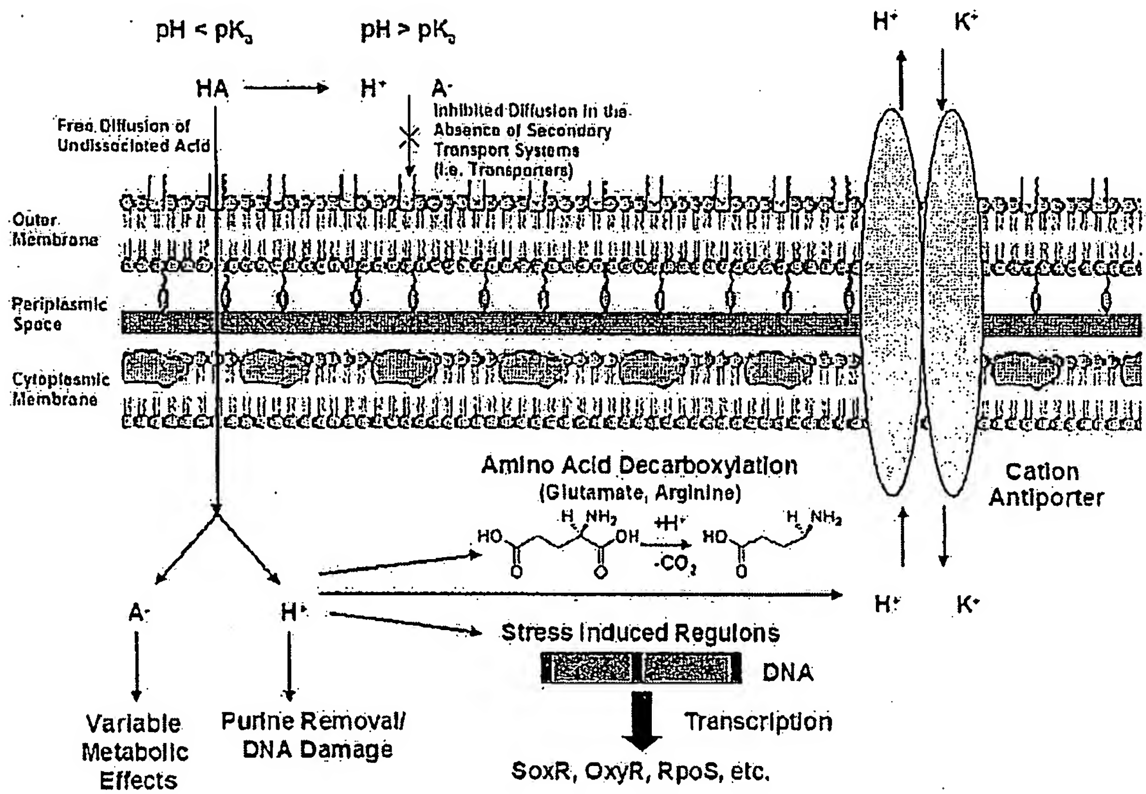

Figure 2
An overview of organic acid toxicity and tolerance mechanisms in *E. coli*. Diffusion of undissociated acid molecules can occur freely in acidic medium but is limited to transport systems at neutral or basic pH. The toxic effects associated with organic acids are the result of both anion specific affects on metabolism as well as increased internal proton concentrations. Affects on internal pH are mitigated by transport of protons out of the membrane, consumption of protons by decarboxylation reactions, and, more generally, induction of stress regulons. Anion specific tolerance mechanisms are not well characterized.

tolerance, they are regulated differently and confer varying levels of tolerance.

Although most acid tolerance systems are activated in stationary phase, acid tolerance as low as pH = 3 has been observed in exponential phase *E. coli* grown under aerobic conditions, which is advantageous from a productivity standpoint [30]. Although the underlying tolerance mechanism is not known, such tolerance can be reliably activated by adapting cells at sublethal pH values between 4.3 and 5.8 [53]. *E. coli* that exhibit growth phase tolerance remain viable at pH values on the same order as stationary phase tolerance, however the percent survival is significantly lower. Lin et al. reported 1% survival of the original culture following acid adaption at pH 4.3 followed by acid challenge at pH 3.3 compared to 0.0001% survival for unadapted cultures. This is compared to stationary-phase cultures, which exhibited up to 50% survival.

Three stationary phase acid resistance systems have been studied in the most detail [29,30]. These systems confer the highest levels of tolerance and are believed to be responsible for stationary phase *E. coli* survival when passing through the mammalian stomach. Acid resistance system 1 (AR1) is activated in slightly acidic media (pH 5.5) in the absence of extracellular glucose or amino acids. *E. coli* grown aerobically under these conditions retain viability under acid challenges as low as pH = 2.5 [54]. This

system is also referred to as the oxidative or glucose-repressed system, since the expression of this system is thought to be regulated either directly or indirectly by RpoS and cyclicAMP receptor protein (CRP) [55,56]. Acid resistance system 2 (AR2) is activated in *E. coli* grown in aerobic conditions in acidic complex media. This system requires the presence of extracellular glucose and glutamate and is dependent upon genes encoding glutamate decarboxylase (*gadAB*) and a glutamate:GABA antiporter (*gabC*) [30]. Under such conditions, *E. coli* have been demonstrated to exhibit acidic-resistance up to a pH of 2. The mechanism involves the expenditure of excess cytoplasmic protons during amino acid decarboxylation reactions (see Figure 2), thus raising the internal pH [54,55]. Acid resistance system 3 (AR3) parallels the mechanisms of AR2 with several slight deviations [30,54,55]. AR3 is activated under anaerobic conditions, in complex media with added glucose. It also involves amino acid decarboxylation reactions to lower the internal pH, but requires extracellular arginine in place of glutamate. AR3 also requires increased expression of arginine decarboxylase and an arginine: agmatine antiporter for increased acid tolerance.

Finally, several general acid tolerance mechanisms that regulate the physical properties of the membrane or the effectiveness of ion transport have been identified. These active responses, or those that occur as a result of the cell's ability to sense pH changes, are independent of growth and are induced by pH shifts as small as 0.2 pH units [57]. The first response is the ability of the microorganism to adjust membrane properties, such as lipid content, thus effectively changing the proton permeability [57]. Another cellular response to acid shock is the induction of genes responsible for repairing and preventing lethal cellular damage. Specifically, increased expression of the *oxyR* and *soxR* regulatory genes has been observed by transcriptional profiling of acid tolerant phenotypes [45,58]. These systems regulate the removal of damaging oxidizing agents, thus preventing further DNA damage under acidic stress [46]. Finally, acid tolerance can be achieved by adjusting the ionic transporter efficiency, effectively regulating the anion and cation balance as a means of maintaining a constant internal pH [47].

Organic Acid Production in E. coli

Metabolic and genetic engineering, directed evolution, and classic strain selection have all been employed in the development of *E. coli* strains that produce building block organic acids, including lactic-acid, succinic acid, and 3HP [17,25,59,60]. Improved titers have been achieved due to optimization of fermentation conditions and relavant pathways utilized. However, titer limitations exist when fermentation is carried out in unbuffered media, which allows the pH to acidify due to increased acid concentration. Alternatively large amounts of base titrant are required to raise the pH of the media during the organic acid production leaving the final acid molecule in the undissociated form. Following production under these conditions, large volumes of acid must be added to recover the acid in the protonated form. Metabolic and genetic engineering of acid tolerance into production strains, making fermentation at a pH less than the pKa of the acid produced possible, would circumvent the need for the additional consumption of acid and base titrants, and thus lower the overall production cost. Similarly, engineering strain fitness to increase productivity at a decreased pH would improve productivity and reduce base consumption.

Lactic acid production is one of the most successful examples to date of the engineering of large volume chemical production in *E. coli*. *E. coli* was selected as a favorable host strain due to its ability to consume both pentose and hexose sugars and to generate optically pure L-lactic acid, which is the desired product for downstream polylactic acid (PLA) production [61,62]. An effective lactic acid producing strain of *E. coli* was created by induced expression of the L-specific lactic acid dehydrogenase (LDH) gene from *Streptococcus bovis*. High titers (50–75 g/L) were observed under controlled pH (pH = 7) and anaerobic conditions. Titers were drastically decreased (10–20 g/L) as the pH was allowed to drop with increasing acid production [59]. However, allowing the pH to fall below the pKa of lactic acid also resulted in decreased concentration of the acid in the undissociated form, which facilitated the subsequent isolation of the protonated acid. Interestingly, the choice of host strain made a significant difference in lactic acid production [59]. Those constructed from an *E. coli* B strain showed a titer of almost twice that produced from K12 derivatives. The increased production was attributed primarily to differences in the native growth characteristics rather than increased acid tolerance.

Economically competitive titers of succinic acid have also been achieved in *E. coli*. Strains were engineered to limit flux to other anaerobic byproducts normally formed during fermentation [60]. Specifically, succinic-acid production was optimized by redirecting the metabolic flux at the pyruvate node away from lactate and formate through inactivation of the pyruvate-formatelyase and lactate dehydrogenase [60,63]. The maximum yield in succinic acid production was approximately 50 g/L in pH controlled cultures. However, similar to lactic acid studies, succinic acid production was significantly repressed when pH was not kept at neutral levels.

A final example of metabolic engineering organic acid production in *E. coli* was reported by Cargill in 2001 [17]. Suthers and Cameron engineered a 2-step glycerol to 3HP

pathway in *E. coli*. Glycerol was first converted to 3HPA via a glycerol dehydratase enzyme (*dhaB* – isolated from *Klebsiella pneumoniae*). 3HPA was then converted to 3HP via an aldehyde dehydrogenase (*ald*). This first pathway was not ideal for several reasons including a very low reported titer (0.2 g/L), the use of the more expensive glycerol as opposed to glucose, and the generation of the highly toxic 3-HPA (reuterin) compound. Selifinova et al. later proposed five additional pathways for the production of 3-HP directly from glucose in *E. coli* [36]. Results for each of such pathways have yet to be reported. One issue that has yet to be addressed is how to fulfill the desire to produce 3-HP at a pH below the pKa = 4.51 of 3-HP, which would lessen the dependency on large volumes of base titrant to retain neutral pH at high titers.

Metabolic engineering of *E. coli* organic acid tolerance represents an important future opportunity. As discussed above, *E. coli* possess several systems for surviving pH as low as 2.0, which is much lower than what is required for an economical biorefining process. Since induction of these systems is well characterized and the relevant genes are known in many cases, future efforts might be better focused on the development of multi-stage fermentations that allow for generation of biomass prior to induction of acid tolerance and, ultimately, acid production. Future genetic engineering efforts might focus on engineering tolerance against the less well characterized metabolic effects associated with increased organic acid anion concentrations. For example, the addition of acetate, benzoate, and propionate to culture media at a concentration of 8 mM has been observed to inhibit growth of *E. coli* up to 50% [35]. The acetate inhibition is thought to be caused by limited methionine pools combined with increasing concentrations of homocysteine, a toxic intermediate, due to inactivation of a key enzyme in the methionine synthesis pathway, which can be countered by the addition of methionine to the media. This finding established that growth inhibition is the result of both of lowered pH and specific anionic effects, which decreases the activity of key enzymes. Thus, engineering tolerance to specific organic acid anion effects by increased expression of inhibited enzymes could aid in increasing overall process productivity.

Conclusion

Organic acids are a valuable sector of the industrial chemical market, which have already been successfully produced through microbial fermentation. However, product titers have been variable, ranging from less than 1 g/L to concentrations cost competitive with current petrochemical production processes. These fermentation processes have been limited in *E. coli* due to product and intermediate toxicity. Toxicity is directly measured by growth inhibition, which specifically decreases productivity. This review highlighted what is known about organic-acid toxicity and tolerance mechanisms in *E. coli*. Specifically, *E. coli* are growth inhibited by the increase in both proton and associated anion concentrations that are characteristic of organic-acid production processes. While several acid-tolerance mechanisms have been characterized in *E. coli*, anion specific mechanisms require additional study. Thus, future metabolic engineering efforts that seek to improve understanding of these issues within the context of organic-acid biorefining applications should prove useful.

Additional material

Additional File 1
Table 1: Organic acids for platform biorefining applications. (see references [64,65])*
Click here for file
[http://www.biomedcentral.com/content/supplementary/1475-2859-4-25-S1.doc]

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